DEFINING THE MOLECULAR AND PHYSIOLOGICAL ROLE OF LEAF CUTICULAR WAXES IN REPRODUCTIVE STAGE HEAT TOLERANCE IN WHEAT

A Dissertation

by

SUCHISMITA MONDAL

Submitted to the Office of Graduate Studies of
Texas A& M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2011

Major Subject: Plant Breeding

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Approved by:

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ABSTRACT

Defining the Molecular and Physiological Role of Leaf Cuticular Waxes in Reproductive Stage Heat Tolerance in Wheat. (May 2011)

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In wheat, cooler canopies have been associated with yield under high temperature stress. The objectives of this study were, i) to understand the role of leaf cuticular waxes as physiological adaptive mechanisms during reproductive stage high temperature stress ii) define quantitative trait loci (QTL) regulating leaf cuticular waxes and determine its link with the QTL for reproductive stage heat tolerance iii) define stable QTL associated with leaf cuticular waxes and yield stability across environments.

For the first objective, thirteen wheat cultivars were subjected to a 2-day heat treatment at 38°C at 10 days after pollination (DAP). Leaf cuticular waxes, canopy temperature depression and stomatal conductance were estimated during high temperature stress. At maturity the percent reduction in yield components in each cultivar was calculated. The wheat cultivars 'Kauz' and 'Halberd' had significantly high leaf cuticular wax content of 2.91mg/dm^{-2} and 2.36mg/dm^{-2} respectively and cooler canopies. Leaf cuticular waxes were significantly correlated with leaf temperature depression and reduction in yield components.

A set of 121 recombinant inbred lines (RIL) population derived from the cross of heat tolerant wheat cultivar ‘Halberd’ and heat susceptible wheat cultivar ‘Karl 92’ was utilized for QTL mapping. The RIL population received a 2-day short-term high temperature stress at 38°C at 10DAP in 2008 and a long-term high temperature stress at 38°C from 10DAP until maturity in 2009 in the greenhouse. The RIL population was also planted in College Station, Texas in 2009 and 2010 and in Uvalde, Texas in 2010. Leaf cuticular wax was estimated at 10DAP and leaf/spike temperatures were recorded during grain filling. Yield components were estimated after harvest. Heat susceptibility indexes for main spike yield components were estimated in the greenhouse.

Overall ten significant QTL were identified for leaf cuticular waxes each explaining 8-19% of the variation respectively. Stable QTL for leaf cuticular waxes were located on chromosome 5A and 1B and co-localized with QTL for leaf/spike temperature depression and HSI for kernel weight and single kernel weight of main spike. Another QTL on chromosome 1B contributed by Karl92 was found in the greenhouse and field environments and co-localized with a previously identified QTL on 1B for spike non-glaucousness. The results suggest that leaf cuticular waxes may reduce leaf temperatures and improve adaptation during high temperature stress.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Bread and durum wheat (*Triticum aestivum* L.) occupy approximately 200 million ha globally, thus wheat is one of the most wide spread cereal in terms of area planted with a production of 646 million tons worldwide in 2010 (FAO 2010). Increased wheat production in the last 40 years has made a major contribution to the global food security. The increase in wheat productivity resulted from the efforts to develop improved varieties with a dwarf stature, increased yield and durable resistance to abiotic and biotic stress. Global climate changes resulting from increased warming elevated atmospheric CO2 and altered precipitation patterns are influencing wheat production worldwide. Simulation studies by IPCC FAR (2007) report that yield may increase in the mid to high latitudes if mean temperatures increase by 1-3°C, though any further increase in the temperature in temperate regions will result in yield losses. It further reports that in the subtropical and tropical regions wheat performs near its maximum tolerance limits and so any small increase in temperatures (1-2°C) will reduce yield (Hodson and White 2009).

In the Great Plains of the United States high temperature stress generally occurs during reproductive development and grain filling (Assad and Paulsen 2002). The temperatures may increase up to 30 to 35°C during grain development and maturation.

This dissertation follows the style of Euphytica.

Increasing temperatures reduce both grain weight and grain number in wheat leading to yield losses (Ferris et al. 1998). Assad and Paulsen (2002) also reported that improvement in high temperature tolerance is one of the important factors that lead to the increased yields in wheat from 1874-1994 in US Great Plains, though many of the current hard red winter wheat varieties grown in Great Plains still show susceptibility in their inability to maintain yield stability and quality under high temperatures stress (Hays et al. 2007a).

Growth rate is accelerated at high temperatures while the grain filling duration is reduced which leads to yield losses (Lawlor and Mitchell 2000). High temperatures also affect the source sink relationship by reducing the rate of photosynthesis and promoting leaf senescence. Plants employ various stress adaptive physiological mechanisms to survive under elevated temperatures. Increased photosynthetic rate, membrane thermostability, leaf chlorophyll content and flag leaf stomatal conductance are some of the physiological responses that have been associated with yield under high temperature stress in wheat (Reynolds et al. 1994). An enhanced understanding of physiological and genetic basis of adaptation in conjunction with molecular approaches will enable us to tackle the problem of increasing temperatures. A discussion on the effects of high temperature stress, the adaptation and genetics of heat tolerance will follow.

1.1. Effects of high temperature stress on crop development and yield

High temperature is a major abiotic stress affecting agronomic and quality characteristics in wheat. The developmental stage at which the plant is exposed to high temperature

stress determines the severity of the effects. High temperature during vegetative stages affects the leaf gas exchange properties (Wahid et al. 2007). Heat stress during reproductive stages cause abortion of floral buds, pollen and anther sterility and restricted embryo development that result in reduction in grain number and yield. In wheat both grain number and weight are sensitive to high temperature stress (Ferris et al. 1998). The effects of high temperature stress also depend on the intensity and duration of stress. Grain weight was decreased by 30% under moderate temperature stress (30°C) (Zahedi et al. 2003). Heat stress for 10 days at 35/20°C reduced kernel weight and kernel number by 29% and 36% respectively (Assad and Paulsen 2002). Since kernel number and kernel weight are the primary components of grain yield, reduction in either component leads to reduced yield (Fischer 1993). The rate and duration of grain filling are both influenced by high temperature stress. Weigand and Cueller (1981) reported a linear reduction of 3 days in grain filling for every 1 °C increase in temperatures within the range of 20-30°C. Grain weight reduces linearly with increased duration of heat stress. However, the first day of stress results in more loss in grain weight than an extended period of stress. Thus a short period of very high temperatures causes proportionally more damage to yield than an extended period of very high temperatures (Stone and Nicolas 1994). Yield losses up to 23 % have been reported in response to 4 days of high temperatures (>32°C) (Randall and Moss 1990). The rate of grain filling also declines with the increase in temperatures above 30°C (Tashiro and Wardlaw 1989).

High temperature during grain filling alters the physico-chemical properties of wheat flour and affects the dough strength and baking quality characteristics. Starch

synthesis is highly sensitive to high temperatures due to the susceptibility of soluble starch synthase (Viswanathan and Khanna-Chopra 2001). Since starch accounts for 70% of the grain weight, a reduction in grain weight under high temperature is primarily due to reduced starch accumulation (Bhullar and Jenner 1985). There is a concomitant increase in protein content in the grain under high temperatures due to reduced starch accumulation (Stone and Nicolas 1998). Although protein synthesis is less sensitive to high temperature stress, an increase in production of monomeric proteins was found under high temperatures stress that lowered the glutenin to gliadin ratio resulting in weak dough which was not suitable for bread (Blumenthal et al. 1995; Castro et al. 2007)

1.2. Effects of high temperature stress on plant physiological components

Plants biomass and yield are proportional to the levels of crop photosynthesis (Loomis and Williams 1963). Elevated temperatures cause an imbalance between photosynthesis and respiration; the rate of photosynthesis decreases whiles the rate of respiration increases. Photosynthesis in wheat is stable at a temperature range of 15 to 30°C. As the temperature rises over 30°C the rate of photorespiration increases more than rate of CO_2 fixation, altering the partitioning of assimilates to reproductive sinks. At temperatures higher than 40°C irreversible damage occurs to the photosynthetic apparatus due to deactivation of PSII. Rubisco also plays a pivotal role in regulation of photosynthesis. High temperature stress inhibits the ability of Rubisco activase to activate Rubisco thereby limiting photosynthesis. At high temperatures the Rubisco oxygenase activity increases due to increased solubility of oxygen relative to carbon dioxide thus leading to higher rates of photorespiration. Under normal temperatures photosynthesis declines

during grain filling, but at high temperatures, reduced photosynthesis causes early senescence reducing the grain filling duration and yield (Wardlaw 1974). As such, it is important for the plants to maintain an optimal leaf tissue temperature for normal growth and development. Transpirational cooling has been associated with reduction of leaf temperatures under well-irrigated conditions.

1.3. Tolerance to high temperature stress

Adaptation mechanisms and stress tolerance mechanisms are important for maintaining yield stability and grain quality. Plants may manifest different heat tolerance mechanisms such as long-term evolutionary phenological and morphological adaptations and short-term avoidance mechanisms or acclimation mechanisms such as changing leaf orientations or transpirational cooling (Majoul et al. 2003; Wahid et al. 2007). Exposure to high temperature stress triggers the signaling processes and transcriptional controls to activate the stress responsive mechanism. In response to high temperature stress the expression of heat shock proteins (HSPs), ubiquitin, late embryogenesis proteins and osmoprotectants are stimulated (Wahid et al. 2007). The HSPs function as chaperones and/or proteases that minimize the effects of high temperatures at cellular and molecular levels (Rampino et al. 2009). Increase in accumulation of HSP transcripts under high temperature stress has been reported in wheat (Rampino et al. 2009). A co-segregation analysis study in winter wheat also suggested the involvement of heat shock proteins in thermotolerance (Joshi et al. 1997). The HSPs are a family of proteins consisting of three classes; HSP90, HSP70 and low molecular weight HSPs. The variation in expression of individual HSPs may result in genetic variability for thermotolerance.

Another important element of high temperature tolerance is membrane lipid saturation. Increased heat resistance in a mutant wheat line was associated with increased levels of lipid compounds linolenic acid and 3-hexaldeconic acid (Behl et al. 1996). High temperature alters the tertiary and quaternary structures of membrane proteins enhancing the permeability of membranes and increased loss of electrolytes. Membrane thermo stability has been expressed in terms of electrolytic conductance and measured by the cellular membrane stability assay (CMS) and tetrazolium triphenyl chloride (TTC) assay. A significant correlation has been observed between the CMS and yield under high temperature stress in wheat (Blum et al. 2001). The TTC assays were also found to be reliable assays for heat tolerance due to their association with membrane stability and high heritability (Ibrahim and Quick 2001a, b).

1.4. Adaptation to high temperature stress

The adaptive mechanisms at high temperatures include increased transpiration, reflective hairs, leaf waxes, leaf orientation and size. A study conducted in a high-yielding environment in Mexico revealed that leaf photosynthetic rate, leaf conductance and CTD were associated with yield in a set of eight spring bread wheat lines (Fischer et al. 1998). Leaf transpiration under high temperature conditions reduces leaf surface temperature. For wheat grown under adequate moisture transpirational cooling may reduce leaf temperatures to as low as 8°C below ambient temperature (Reynolds et al. 1994). Canopy temperature depression has been suggested as a heat escape mechanism (Cornish et al. 1991). In both warm and temperate environments, CTD showed a high correlation with yield and had high values for heritability (Reynolds et al. 1998). Due to

the ease of measuring CTD it has been identified as a potential tool for indirect selection (Reynolds et al. 1994, Amani et al. 1996). CTD can be directly or indirectly affected by a number of physiological and morphological features (Reynolds et al. 2001). CTD is associated with vapor pressure difference, which depends on relative humidity and air temperature. Therefore CTD may not be useful for selection under cool/humid conditions. Variation in morphological features (degree of waxiness, leaf size and awns) may lead to differences in energy absorption, latent heat flux or a combination of factors that may result in cooling of canopies (Ayeneh et al. 2002). There are a few studies that report the association of morphological traits with temperature depression. Blum (1986) reported that the presence of awns contributed to cooler canopy temperatures in wheat. Though leaf rolling is an adaptive feature in drought stress conditions, it increases leaf temperatures due to reduced transpiration (Ayeneh et al. 2002). Cuticular wax load affects the epidermal conductance rate and also influences the ultra-structure and topology of the leaf surface (Cameron et al. 2005). Although it has been suggested that leaf cuticular waxes may be associated with canopy temperature depression, no studies have been conducted for it.

1.5. Leaf cuticular waxes

The plant cuticle serves an important function in reflecting excess photosynthetic energy and heat generating infra-red radiation. The cuticle is a thin hydrophobic layer that covers primary aerial plant surfaces such as leaves, fruits and stems (Fig. 1). The thickness of cuticle layer varies between species however there are three distinct layers in all species. The cuticular layer is in contact with the cell wall, above that is the cuticle

proper. Coating the surface of cuticle proper are epicuticular waxes (Bird 2008). In wheat it is difficult to distinguish between cuticular layer and cuticle proper (Jeffree 2006). Cuticle layer is composed of cutins and waxes. Cutin forms the framework of the cuticular matrix with the waxes embedded in this cuticular matrix (intracuticular) and also deposited on the surface as epicuticular waxes. The waxes are composed of long-chained hydrocarbons, ketones, esters, aliphatic alcohols, fatty acids and aliphatic aldehydes. Cuticular waxes in plants provide a protective barrier to abiotic and biotic stresses. Sorghum has the highest amount of leaf wax among cereals (~52.7mg/g) while durum wheat has a range of 25-37.5 mg/g of leaf depending on locations (Burrow et al. 2008). A study by Richard et al. (1986) estimated high epicuticular wax amounts in wheat flag leaves at anthesis. Both leaf conductance and the topology of leaf surface affect yield (Johnson et al. 1983). Leaf epicuticular waxes directly interact with the environment. A study on effect of high day and night temperature in velvet Mesquite revealed an increase in total wax content when grown at high day and night temperatures (Hull 1958).

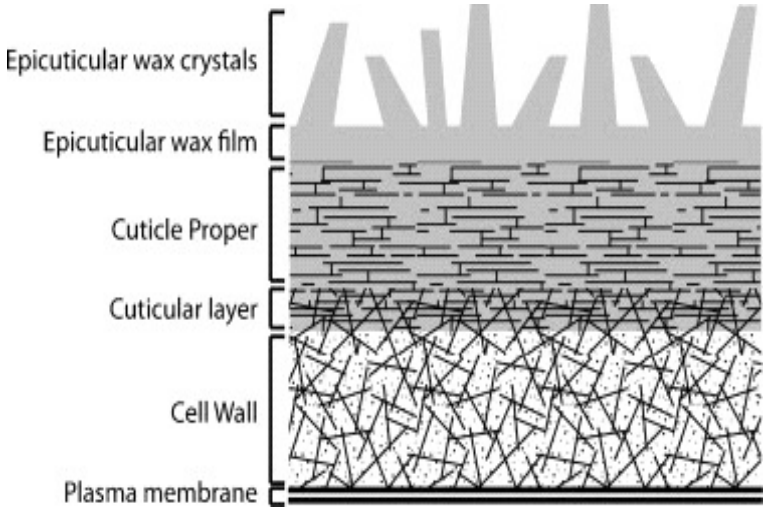


Fig. 1 Schematic presentation of leaf cuticle structure in plants (Bird 2008)

Epicuticular wax imparts a bluish green color to the leaf that is known as glaucousness. Leaf waxes may affect leaf spectral properties to the extent that the net radiation and leaf temperature are reduced (Blum 1975). Glaucousness in wheat has been associated with reduced leaf temperatures and delayed heat induced leaf senescence (Johnson et al. 1983). Studies by Johnson et al. (1983) suggested that glaucousness may not be an indicator of the quantity of wax and thus both quantitative and qualitative measurement of leaf wax is important. Differences in leaf surface reflectance are observed between glaucous and non-glaucous lines and with increases in leaf cuticular waxes under drought stress conditions (Uddin and Marshall 1988). Leaf reflectance increases linearly with the increase in epicuticular wax content in wheat. Spectral reflectance indices provide a non-destructive, instantaneous and quantitative assessment of a crop's ability to intercept radiation and photosynthesize (Ma et al. 1996). Spectral

reflectance indices, photochemical reflectance index [PRI= (R 531- R570)/ (R531+R570)], simple ratio (SR=NIR/VIS), water index (WI=R970/R900) and R550 are suitable estimators for yield in durum wheat (Royo et al. 2002). Total reflectance and spectral indices will be used as a tool to study physiological changes in response to high temperatures in wheat.

The leaf wax biosynthesis process consists of three stages. In the first stage the C16 and C18 fatty acids are synthesized, the second stage involves elongation of the C16 and C18 fatty acids to very long chain fatty acids (VFLCAs), and in the final stage of wax production the VFLCAs are modified to various wax products such as alcohols, esters, ketones, and aldehydes. The wax biosynthesis process has been localized to the endoplasmic reticulum (ER) but the export of waxes from epidermal cells to the plant surface is not well understood. Studies suggest that ATP binding cassette (ABC transporters) and lipid transfer proteins (LTP) are involved in cuticular wax transport (Pighin et al. 2004, Hoh et al. 2005). The expression of ABC transporters and LTPs increases under heat stress conditions in wheat (Hays et al. 2007b) suggesting their possible role in the transport of cuticular waxes. Various genes controlling wax production and movement have been identified in Arabidopsis, Sorghum, maize, barley and rice. In Arabidopsis at least 21 loci involved in a wax accumulation pathway (ECERIFERUM and CER loci) have been identified because mutants with easily distinguishable phenotype were available (Xia et al. 1996). Among the wax-related genes identified CER1, CER2, CER6/CUT1, KCS1, FDH, and WAX2 in Arabidopsis, GL1 and GL8 in maize, GL1 in rice and WXP1 from Medicago encode wax synthesis

and transport related enzymes or proteins, while CER3, GL2, GL15 and WIN1/SHN1 encode regulatory proteins (Islam et al. 2009).

1.6. Genomics in heat tolerance

Wheat is an allohexploid (AABBDD, 2n=6x=42) with a genome size of 16 x 109 bp/1C (Bennett and Smith 1976). Hexaploid wheat evolved from the hybridization of three diploid ancestors, *Triticum urartu* (AA), *Aegilops speltoides* (BB) and *Aegilops tauschii* (DD) respectively (Dubcovsky and Dvorak 2007). Wheat has the largest and complex genomes, 90% of which constitutes non-coding repetitive elements and less than 3% are coding genes (Li et al. 2004). Wheat behaves like a diploid due to the presence of Ph1 gene on chromosome 5B that prevents homeologous pairing (Akhunov et al. 2003). Though wheat has a large genome size (40 times of rice genome) but the gene order and content in each of the 7 homeologous chromosome is conserved (Singh et al. 2007).

Wheat has extensive resource of molecular markers that can be utilized for genetic analysis and marker trait association studies. High-density genetic maps and physical maps are available in wheat (Somers et al. 2004; Xue et al. 2008; Roder et al. 2000). The simple sequence repeat marker/ microsatellite marker (SSR) has been the most widely used marker for genetic mapping studies. The SSR markers are highly polymorphic and transferable across the different genetic backgrounds both within and between species (Hearne et al. 1992). More than 90,000 molecular markers are available in wheat that can be utilized to dissect complex traits and identify important genetic loci regulating these traits. Mapping quantitative trait loci (QTL) in a bi-parental population allows detection of chromosome segments controlling the traits of agronomic interest.

Crop performance is a result of action of several genes and their interaction with environmental conditions. Genetic dissection of quantitative traits controlling adaptive responses of crops to heat stress is essential to understand their regulation and facilitate their manipulation to improve sustainability and stability of yield (Collins et al. 2008). In wheat, a number of QTL mapping studies have reported identifying QTL controlling yield, yield components and agronomic traits (Cuthbert et al. 2008; Kuchel et al. 2007a, Wang et al. 2009) though most studies were not conducted under specific environmental conditions. A few studies have reported genetic loci associated with yield, quality stability and physiological traits under high temperature stress. Two genetic loci controlling grain filling duration under heat stress were detected on chromosome 1B and 5A explaining 23% of the total variation (Yang et al. 2002b). Kuchel et al. (2007b) reported a QTL for yield on chromosome 1B at the same loci as grain filling duration. A recent study identified QTL associated with heat susceptibility and temperature depression on 1A, 5A and 6D in wheat (Mason et al. 2010). Study with di-telosomic lines developed from Chinese Spring wheat cultivars suggested the involvement of long of chromosome 1B and 7D in heat tolerance (O'Mahony et al. 2000). QTL for senescence traits related to heat tolerance were found on chromosome 2A, 6A and 6B, 3A, 3B and 7A.

In wheat glaucousness is under the control of both major and minor genes (Johnson et al. 1983). Cytogenetic studies reveal the presence of a glaucousness gene W1 on chromosome 2BS and the dominant inhibitor gene Iw2 on chromosome 2DS in wheat. Dubcovsky et al. (1997) reported another spike glaucousness inhibitor gene Iw3

located on chromosome 1BL. To date there has been no reports on molecular markers associated with leaf cuticular wax content in wheat.

1.7. Rationale and objectives of the project

High temperature stress causes changes in various physiological and biochemical processes and development of morphological and physiological adaptations will facilitate stress avoidance. Cuticular waxes may function as a stress avoidance mechanism by radiating excess energy and reducing leaf temperatures under high temperature conditions. A fundamental understanding of the molecular and physiological basis of improved adaptation conferred by the presence of leaf cuticular waxes and its variable content and composition to high temperatures does not exist. Thus a detailed understanding of the role of leaf cuticular waxes in heat stress resistance is essential. The primary goal of this proposal is to understand the of role leaf cuticular waxes as a morpho-physiological adaptive mechanism in high temperature stress tolerance and to identify associated genetic loci that improve adaptation to high temperature stress.

Plants use a small portion of the intercepted solar energy in photosynthesis and the remainder is dissipated as heat. Transpiration and increased reflectance are the major avoidance mechanisms plants use to dissipate excess heat. The cuticle plays an important role in reflecting the infrared radiation that is responsible for the heating the leaves. The central hypothesis of this proposal is that cuticular waxes present on the leaf surface reduce leaf tissue temperatures by reflecting excess photosynthetic and heat

generating infrared radiation and will improve adaptation of wheat lines to high temperatures.

The specific objectives that will be used to test this hypothesis are:

Objective1. Characterize the flag leaf cuticular wax content and understand its association with the other physiological adaptive mechanisms (CTD and stomatal conductance) and yield under high temperature stress. This objective follows the hypothesis that presence of leaf cuticular waxes will radiate excess heat generating radiation and reduce leaf temperatures thereby contributing to heat tolerance. This study will be conducted in a greenhouse with a set thirteen wheat cultivars. Heat tolerance will be measured as percent reduction in yield components under high temperature stress and be associated with the morpho-physiological adaptive traits.

Objective2. Defining genetic loci regulating the leaf cuticular wax content and canopy temperature depression, characterizing their relationship with reproductive stage heat tolerance under short-term and long-term heat stress. The hypothesis is that heat tolerance is a quantitative trait and identification of genetic loci regulating the heat adaptive traits will be co-localized with heat tolerance traits. A recombinant inbred line population (RIL) developed from a cross of Australian heat tolerant line ‘Halberd’ and hard red winter wheat cultivar ‘Karl92’ will be characterized for leaf cuticular wax content. Heat tolerance will be estimated based on the stability of the yield components and calculated as heat susceptibility index. Genetic mapping will used to identify QTL associated with leaf cuticular wax content and HSI of yield components.

Objective3. Identify stable loci associated leaf cuticular waxes and canopy temperature depression across field environments and determine their association with yield. The hypothesis is that the stable genetic loci for physiological adaptive traits will improve stability of yield. The RIL population will be analyzed in a multi-environmental trial to identify stable loci for yield and morpho-physiological traits that improve heat tolerance in wheat.

CHAPTER II

ROLE OF LEAF CUTICULAR WAXES AS A PHYSIOLOGICAL ADAPTIVE MECHANISM IN REPRODUCTIVE STAGE HEAT TOLERANCE

2.1. Introduction

Wheat is one of the most important food grain sources in the world with production area of 200 million ha globally and production of 646 million tons worldwide in 2010 (FAO 2010). Increased wheat production in the last 40 years has made a major contribution to the global food security. The increase in wheat productivity resulted from the efforts to develop improved varieties with increased yield and durable resistance to abiotic and biotic stress. Global climate change resulting from increased warming elevated atmospheric CO_2 and alterations in precipitation patterns, are influencing wheat production worldwide. Simulation studies by IPCC FAR (2007), reports that yields may increase in the mid to high latitudes if there is a moderate temperature increase of 1-3°C, however any further increase in temperature will result in yield losses. It further reports that in the subtropical and tropical regions, wheat performs near its maximum tolerance limits and any small increase in temperatures (1-2°C) will significantly reduce yield (Hodson and White 2009).

In the Great Plains of the United States high temperature stress generally occurs during reproductive development and grain filling (Assad and Paulsen 2002). The temperatures may increase up to 30 to 35°C during grain development and maturation. These temperatures reduce both grain weight and grain number in wheat lead to reduced

yields (Ferris et al. 1998). Assad and Paulsen (2002) also reported that improvement in high temperature tolerance is one of the important factors that led to the increased yields in wheat from 1874-1994 in US Great Plains, however many of the current hard red winter wheat varieties grown in Great Plains still show susceptibility in their inability to maintain yield and quality under high temperatures stress (Hays et al. 2007a).

Plants employ various stress adaptive physiological mechanisms to survive under elevated temperatures. Reduced photosynthetic rate and flag leaf stomatal conductance are some of the physiological responses that have been associated with yield under high temperature stress in wheat (Reynolds et al. 1994). Canopy temperature depression (CTD) is the manifestation of crop metabolic and physiologic response to elevated temperatures. Due to the ease of its measurement CTD is an excellent tool for screening for heat tolerant germplasm. Although the physiological basis of the association of CTD and yield is not known, CTD is a function of evapotranspiration and an integration of a number of morpho-physiological and metabolic processes such as stomatal conductance, leaf morphology, photosynthetic rate and vascular capacity (Reynolds et al. 1994).

The effect of leaf morphological differences on CTD and yield under high temperature stress is largely unknown in wheat. A study by Araus et al. (1993) showed that an erect leaf trait is associated with increased canopy temperature depression but not with yield itself. Leaf cuticular waxes have been associated with reduced leaf temperature, increased transpiration efficiency and yield under drought stress in wheat (Richards et al. 1986; Blum 1975). However, the effect of leaf cuticular waxes on CTD under high temperature conditions has not been studied in wheat. The presence of leaf

cuticular waxes may contribute to heat tolerance and lower leaf temperatures by reducing both the non-photosynthetic and photosynthetic wavelength heat load. The goal of the present study was to characterize wheat cultivars for their leaf cuticular wax content and understand its association with the physiological parameters such as CTD, stomatal conductance and yield under high temperature stress.

2.2. Material & methods

2.2.1. *Plant material and culture*

Thirteen wheat cultivars consisting of seven spring and six winter wheat types were grown in a greenhouse in 2008 and 2009. The cultivars included in the study, their origin country of origin and adaptation types is given in Table 1.

Table 1 The sources and adaptation of thirteen wheat genotypes evaluated in the study

Cultivar	**Origin Country**	**Adaptation**
Seri M82	Mexico	Spring
Seite Cerros (7 C)	Mexico	Spring
Halberd	Australia	Spring
Kauz	Mexico	Spring
Diebre	Sudan	Spring
Australith	Israel	Spring
Fang 60	Thailand	Spring
Karl 92	USA	Winter
TAM 110	USA	Winter
Cutter	USA	Winter
Jagger	USA	Winter
MIT	USA	Winter
Ogallala	USA	Winter

2.2.2. *Plant culture*

Winter wheat cultivars were germinated in petri dishes and vernalized for six weeks at 4°C. Twenty replications of each cultivar were planted in 12 x 15cm pots filled with 1:3 peat: sand mixture and arranged in a completely randomized design in the greenhouse. Plants were fertilized with 5 grams of Osmocote™ and were supplemented with Peters™ (20:20:20) at the recommended rate once every two weeks starting from a two-leaf stage. Plants were grown at a 20°C /18°C day / night temperatures cycle with a 12h photoperiod from 6 AM to 6 PM, under natural sunlight with 600 μmol m^{-2} s^{-1} PAR supplemental light. The first pollinated spike for each plant was tagged for day of pollination upon emergence of the anther from the pollinated spikelet.

2.2.3. *Cultivar treatment*

Two growth chambers with identical light conditions were set up for the control and heat treatment. At 10 days after pollination half the replications of each cultivar received a heat stress treatment for two days in a growth chamber at 38°C/ 18°C day/night temperatures respectively. The second half was moved to the control growth chamber maintained at control conditions of 20°C/ 18°C day/night temperature respectively for two days. Day length and light conditions in the greenhouse were maintained in the growth chambers. After treatment the plants were moved back to the greenhouse and grown until maturity.

2.2.4. *Leaf wax quantification*

Leaf wax content was extracted and estimated from the flag leaf via a colorimetric technique described by Ebercon et al. (1977). The flag leaf samples were collected from each cultivar at 10DAP, 12 DAP and 15 DAP. Six leaf discs of 1 cm in diameter were taken from each flag leaf sample and placed in a vial. There were six replications of each cultivar at each date. The leaf wax was extracted with HPLC grade chloroform for 30 sec and vacuum dried. The extracted wax was oxidized with 300µl acidic Potassium dichromate ($K_2Cr_2O_7$) in a water bath for 30 minutes. After cooling, 700µl of deionized water was added to each sample and allowed to develop color for an hour. The optical density of each sample was recorded at 590nm. A standard curve was prepared following the similar procedure of wax extraction from the thirty random wheat flag leaves. The extracted leaf wax was weighed and redissolved in chloroform to prepare known concentration aliquots by the serial dilution technique. The resulting linear standard curve was used for estimating leaf wax quantity.

2.2.5. *Scanning electron microscopy*

The flag leaves from all the wheat cultivars were examined with a JEOL 6400 Scanning Electron Microscope (SEM) at the TAMU Microscopy Lab. Fresh leaf samples were collected at 10 DAP from each cultivar and processed according to the method described by Ellis and Peddleton (2007). The leaf samples were placed on the double-sided adhesive tape on the aluminum stubs in a vapor chamber. The leaf samples were vapor coated by placing a small plastic container with osmium tetroxide and ruthenium

tetroxide in the vapor chamber. The vapor stabilized leaf samples were then sputter coated with Au/Pd and examined.

2.2.6. *Leaf reflectance*

Flag leaf spectral reflectance measurements were taken by a portable spectrometer (Unispec-SC, PP Systems, Boston, MA). The spectrometer has a built in light source and can detect reflected light in a range of 310-1100nm that covers the visible and near infrared (NIR) region of the spectrum. Spectral measurements were taken at 10DAP and 12DAP between 11AM to 2PM in three replicates along the length of the flag leaf of each wheat cultivar. The mean of the three readings were used to estimate the spectral reflectance indices (Table 2).

Table 2 The formulae, function and references of different spectral reflectance indexes estimated

Spectral Reflectance Indices	**Formula**	**Function**	**References**
Normalized difference vegetation index (NDVI)	$(R_{780} - R_{670})/ (R_{780} + R_{670})$	Estimation of canopy photosynthetic area	Penuelas et al. (1993)
Simple ratio (SRI)	R_{980} / R_{680}	Estimation of canopy photosynthetic area	Penuelas et al. (1998)
Water index (WI)	R_{970} /R_{900}	Canopy water status	Penuelas et al. (1993)
Photochemical reflective index (PRI)	$(R_{531} - R_{570}/ (R_{531}+ R_{570})$	Radiation-use efficiency	Penuelas et al. (1995)

2.2.7. *Physiological measurements*

Flag leaf temperatures were taken between 11a.m and 2p.m on each day of the heat and control treatments with a handheld infrared thermometer (Model AG-42, Teletemperature Corp, Fullerton, CA). The measurements were taken at an angle of 45° to the horizontal. The leaf temperature depression (TD) was estimated as the difference between the leaf temperature and the temperature of the heat/control chamber. The stomatal conductance measurements were taken between 11AM and 2 PM with a handheld porometer (Decagon Services Inc, Pullman, WA) under both control and heat stressed conditions.

2.2.8. *Phenological measurements*

At maturity the wheat cultivars were harvested and threshed separately for primary spike and the all other spikes were counted and threshed together. The yield components, kernel number, kernel weight and single kernel weight were determined for the primary spike. Only the yield components from main spike were used in data analysis to control errors in the treatments associated with non-uniform tillering and flowering of secondary tillers (Mason et al. 2010; Yang et al. 2002a) Grain filling duration (GFD) was estimated as the date of pollination until 90% senescence of the main spike. Heat intensity index (HI) or percent reduction (%R) was estimated to evaluate the performance of each cultivar under high temperature stress (Porch 2006). A cultivar with high HI index or % R was susceptible to high temperature stress.

It was calculated for each yield component as

HI or % R = $(Y_C-Y_H)/Y_C$

Where, Y_C and Y_H are the mean of each yield component for each cultivar under control and heat stressed conditions respectively.

2.2.9. *Statistical analysis*

Statistical analyses were conducted using SAS v 8.2 (SAS Institute Inc., Cary, NC, USA). The generalized linear model (GLM) was used for analysis of variance and the means were compared using Fischer's least significant difference. A combined analysis of variance was also done using GLM procedure considering genotype and year as fixed effects. Pearson's correlations were estimated for determining the association between leaf cuticular waxes and the physiological and phenological responses under high temperature stress.

2.3. Results

2.3.1. *Leaf wax morphology*

The flag leaf cuticular wax morphology was investigated by SEM imaging. The cuticular wax on the abaxial side of the leaf surface formed platelet like structure (Fig. 2). SEM showed no difference in the wax morphology between the abaxial and the adaxial sides of the flag leaf. Differences were observed in the distribution of the cuticular wax platelets on the flag leaf surface between the wheat cultivars. The wheat cultivar Halberd and Seri has a dense network of epicuticular wax platelets. Karl92 and Australith have a sparse distribution of platelets on its leaf surface. The cross-sectional view of the leaf

surface distinguished the cuticular layer into the epicuticular and the cutin layer in Halberd. On a visual comparison of the cross-sectional view of the leaf surface, Halberd appears to have a thicker cuticular layer than Karl92. The actual dimension of the wax crystals or the cuticular layer was not possible due to the differences in the imaging distance and resolution between the flag leaf samples.

2.3.2. *Leaf wax quantification*

Flag leaf cuticular wax content was estimated from the standard curve (Fig. 3). Significant differences were observed between the wheat cultivars for the flag leaf cuticular wax content at 10DAP in both 2008 and 2009 (Table 3). In both years, Halberd and Kauz had significantly higher leaf cuticular wax content at 10DAP while Australith and Jagger were the lowest in wax content. The estimation of leaf cuticular waxes at 12 DAP and 15 DAP reflects the changes in leaf cuticular wax content during high temperature stress and after stress respectively (Fig. 4). The wheat cultivars responded differentially to high temperature stress. The cultivars Halberd and Seri M 82 had an increase in leaf wax content at 12DAP on exposure to high temperature stress and a subsequent reduction after stress at 15DAP. The leaf cuticular wax content in Ogallala, 7Cerros, Fang 60, Karl 92, Diebre and Australith increased at 12DAP in response to stress and increased further at 15 DAP. The wheat cultivars MIT and TAM110 had no significant changes in their leaf wax content in response to high temperature stress. Leaf cuticular wax content had significant environment and genotype x environment effect at 10DAP, 12DAP and 15DAP (Table 3).

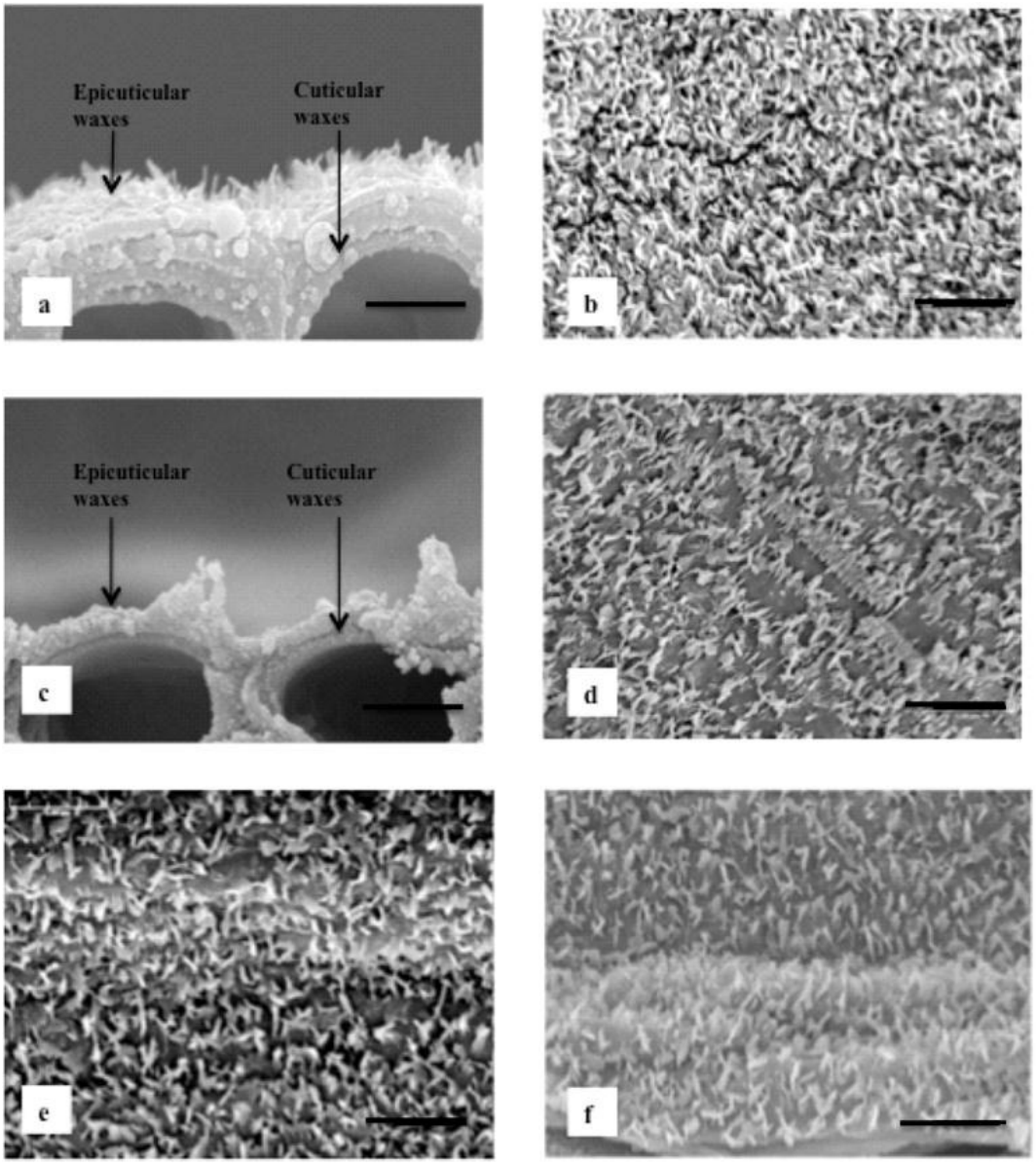


Fig. 2 SEM images of flag leaves collected at 10DAP. The figures a and b are the cross-sectional and top surface view of the flag leaf of wheat cultivar 'Halberd'. The figures c and d are the cross-sectional and top surface view of the flag leaf of wheat cultivar 'Karl92'. The figures e and f are the top surface view of the flag leaf of the cultivars 'SeriM82' and 'Australith'. The epicuticular waxes are present on the leaf surface and arranged in platelet shape. The cross-sectional images show the differentiation of the cuticle layer into epicuticular layer and cuticular layer. Bar (a, b) =15µm, (c, d, e, f) =5 µm

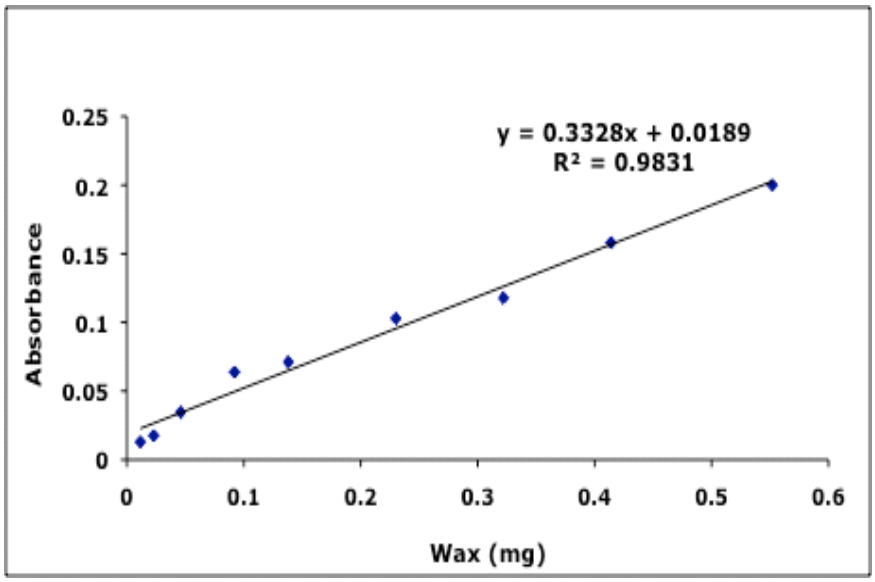


Fig. 3 Standard curve for wheat flag leaf wax estimation

Table 3 Mean square estimates for flag leaf cuticular wax content at 10DAP, 12DAP and 15DAP in the individual years (2008 and 2009) and combined analysis

	Wax 10DAP	**Wax 12DAP**	**Wax 15DAP**	**TD**
2008				
Genotype	1.80*	2.65**	2.61**	3.49*
Error	0.39	0.59	0.14	1.49
2009				
Genotype	2.43**	2.71**	2.15**	1.79*
Error	0.043	0.15	0.51	0.78
Combined analysis				
Genotype	2.34**	4.45**	5.17**	6.07**
Year	3.37**	4.53**	16.36**	2.03
Genotype x Year	0.80**	1.13**	0.99**	8.29**
Error	0.178	0.08	0.28	0.97

DAP –days after pollination TD- Leaf Temperature Depression
* Significant at p=0.05, ** Significant at p=0.01

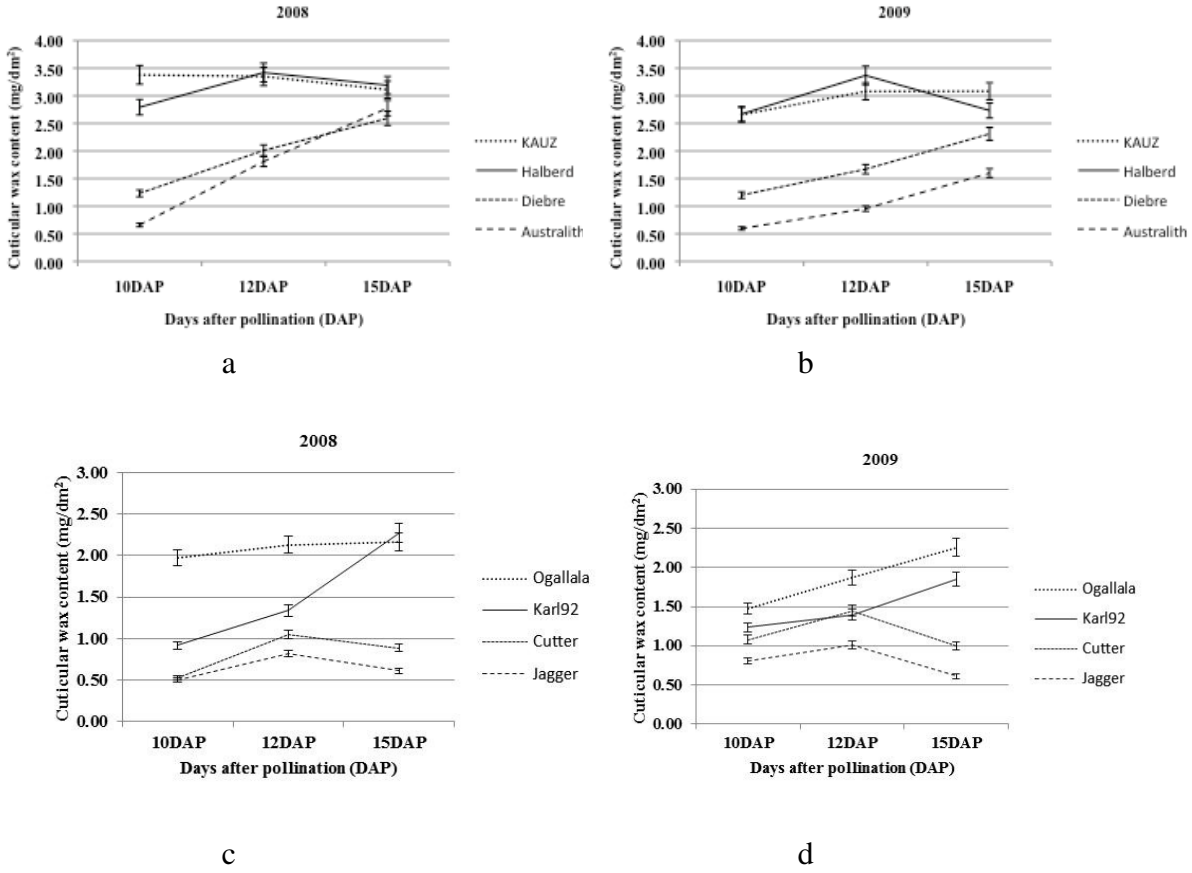


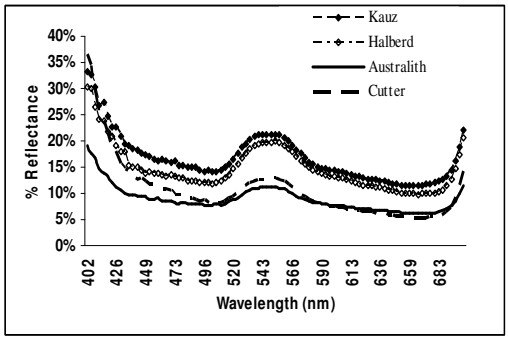
Fig. 4 Flag leaf cuticular wax content in response to high temperatures stress at 10DAP, 12DAP and 15 DAP. The figures a and b display the variation in leaf cuticular wax content in the four spring lines Kauz, Halberd, Diebre and Australith in 2008 and 2009 respectively. The figures c and d display the variation in leaf cuticular wax content in the four winter lines Ogallala, Karl92, Cutter and Jagger in 2008 and 2009 respectively. In 2008, Fischer's LSD (p=0.05) at 10DAP =0.62, 12DAP=1.24 and 15DAP=0.61. In 2009, Fischer's LSD (p=0.05) at 10DAP=0.35, 12DAP =0.810 and 15DAP=0.58

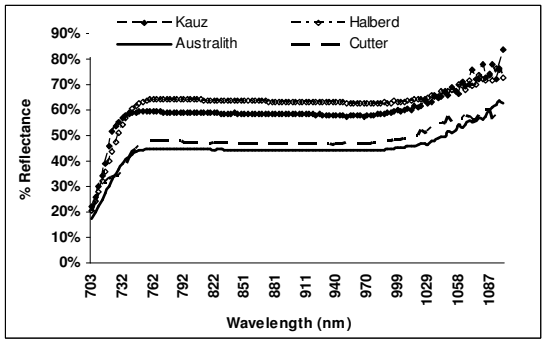
2.3.3. *Leaf spectral reflectance*

Differences were observed in reflectance between the cultivars in the photosynthetic active region and the infrared region (Fig. 5). The wheat cultivars Kauz and Halberd with high leaf wax content had a 5-10% higher average reflectance in the photosynthetic active region than the cultivars Cutter and Australith. Similarly in the near infrared region the lines Kauz and Halberd had a 10-15% higher reflectance than the cultivars Australith and Cutter. Since there were only 3 replications per line for reflectance no significant differences were observed in the reflective indexes NDVI, SRI and WI between the cultivars.

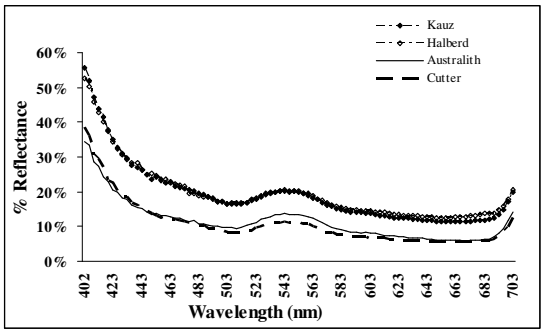
2.3.4. *Leaf temperature depression and stomatal conductance*

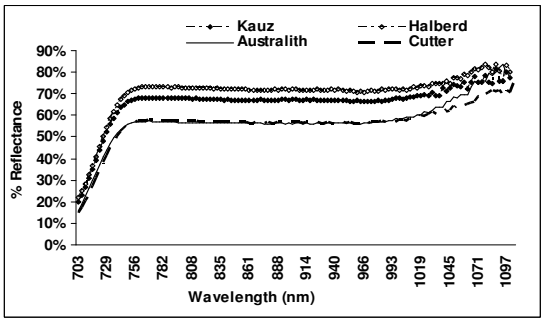
Leaf temperature depression (TD) during high temperature stress was calculated as the difference between the temperature of the heat chamber (38°C) and the flag leaf temperature. Higher leaf temperature depression indicated cooler canopies. The TD in wheat cultivars ranged from 3.2°C to 6.1°C. The genotypic differences in TD were statistically significant (Table 4). The cultivars Halberd, Seri, 7C and Kauz had significantly higher leaf temperature depression (Fig. 6). The wheat cultivars Australith, Karl 92 and Jagger had the lowest leaf temperature depression. Similar results were observed in both the years 2008 and 2009. Interestingly the four winter wheat cultivars Cutter, Ogallala, MIT and TAM 110 grown in the South and Central Texas had significantly higher temperature depression than the two Kansas cultivars Karl 92 and Jagger.





2008 2008





2009 2009

Fig. 5 Percent reflectance over the photosynthetic active region (400-700nm) and the infrared region (700-1100nm) in 2008 and 2009. Reflectance was measured on the abaxial side of the flag leaves in the cultivars Kauz, Halberd, Australith and Cutter. Kauz and Halberd had high leaf wax content while Australith and Cutter were the lowest

Stomatal conductance increased in all wheat cultivars under high temperature stress (Table 4). Differences were observed in the rate of stomatal conductance between the wheat cultivars under heat stress (Fig. 7). The wheat cultivars Australith, Cutter, Fang 60, Jagger, Karl 92, MIT, Ogallala and TAM110 had significantly higher leaf stomatal conductance. Though Halberd and Kauz had higher TD, their stomatal conductance was significantly lower than the other wheat cultivars.

2.3.5. *Yield components*

High temperature stress resulted in significant reduction in yield components in the primary spike (Table 5). Higher reductions in yield components imply susceptibility to high temperature stress. Mean percent reduction in the main spike yield components kernel number per spike (RKnms), kernel weight per spike (RKwms) and single kernel weight (RSkrms) are presented in Table 6 .The wheat cultivars Australith, Cutter, Karl 92, Diebre, TAM110 and Fang 60 had a 10-20% reduction in kernel number (RKnms) and kernel weight of the primary spike (RKwms). 7Cerros, Halberd, Kauz and Seri had significantly less reduction in kernel number and kernel weight of the primary spike. No significant differences were observed for the reduction in single kernel weight of the main spike (RSKrms) between the cultivars in the individual years. Significant genotype by environment effect was observed in all yield components in a combined analysis.

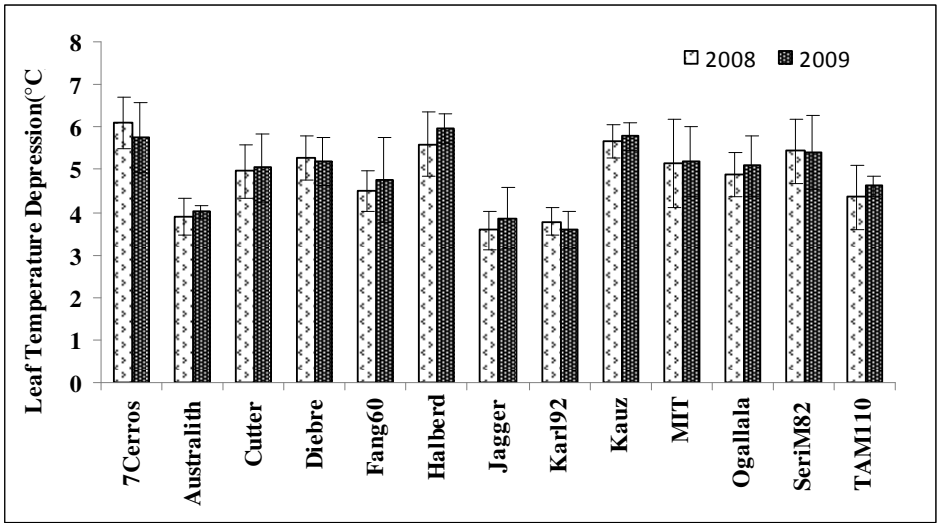


Fig. 6 Leaf temperature depression (TD) in the 13 wheat cultivars under high temperature stress condition in the years 2008 and 2009. Fischer's LSD for TD in the year 2008 =1.59 and year 2009 =1.08 at p=0.05

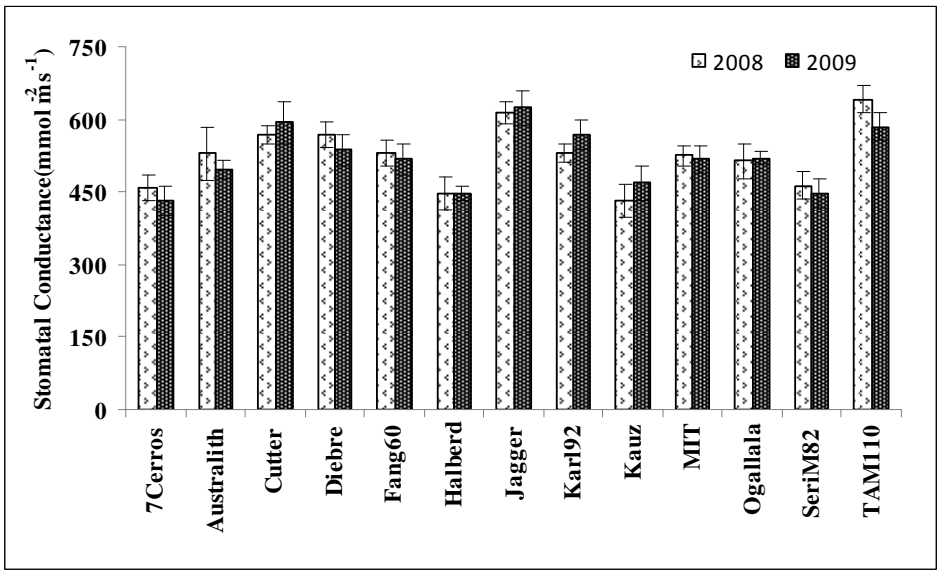


Fig. 7 Leaf stomatal conductance in the 13 wheat cultivars under high temperature stress condition in the years 2008 and 2009. Fischer's LSD for TD in the year 2008 =155.25 and year 2009 =175.3 at p=0.05

Table 4 Mean square estimates for stomatal conductance in individual years and combined analysis during high temperature stress

Individual year analysis				
	Genotype	**Treatment**		**Error**
2008				
Stomatal conductance	180042**	509381**		17684.27
2009				
Stomatal conductance	32952**	143240**		10679
Combined analysis				
	Genotype	**Year**	**Genotype x Year**	**Error**
Stomatal conductance	38869**	616467**	80311**	9953

* Significant at p=0.05, ** Significant at p=0.01

Table 5 Means square estimate for the reduction in kernel number, kernel weight and single kernel weight of main spike in individual years and combined in analysis

	RKnms	**RKwms**	**RSKrms**
2008			
Genotype	0.14**	0.08**	0.01
Error	0.022	0.027	0.08
2009			
Genotype	0.06*	0.10*	0.003
Error	0.018	0.038	0.07
Combined analysis			
Genotype	0.20*	0.28**	0.03
Year	0.185**	0.20	0.25**
Genotype x Year	0.20**	0.18**	0.09**
Error	0.032	0.052	0.0086

Rknms – Reduction kernel number main spike, Rkwms – Reduction kernel weight main spike, RSkwms – Reduction Single kernel weight main spike
* Significant at p=0.05, ** Significant at p=0.01

Table 6 Mean % reduction and Fischer's LSD values for the main spike yield components of the thirteen wheat cultivars evaluated in 2008 and 2009

Cultivars	2008			2009		
	RKnms	**RKwms**	**RSKrms**	**RKnms**	**RKwms**	**RSKrms**
7 Cerros	4.90^{b}	2.75^{ab}	6.04	-4.10^{d}	5.79^{c}	7.81
Australith	12.74^{ab}	10.83^{ab}	3.11	18.23^{a}	17.69^{a}	10.79
Cutter	21.72^{a}	16.29^{a}	7.20	20.89^{a}	17.20^{a}	8.64
Diebre	11.35^{ab}	15.61^{a}	7.96	12.15^{b}	13.62^{ab}	6.88
Fang 60	21.97^{a}	18.25^{a}	3.39	7.57^{bc}	12.17^{ab}	7.58
Halberd	4.32^{b}	4.18^{ab}	-0.73	3.73^{cd}	4.25^{c}	1.07
Jagger	-	-	-	16.25^{ab}	14.38^{ab}	6.65
Karl 92	11.62^{ab}	18.37^{a}	5.31	13.16^{bc}	18.15^{a}	3.36
Kauz	3.98^{b}	4.09^{ab}	3.15	1.42^{cd}	-1.60^{cd}	1.39
MIT	-	-	-	6.56^{c}	12.06^{ab}	7.96
Ogallala	7.85^{ab}	14.97^{a}	3.33	11.76^{bc}	19.35^{a}	8.05
SeriM82	3.92^{b}	-2.16^{c}	0.36	5.53^{c}	1.54^{cd}	-0.81
TAM110	14.28^{ab}	13.64^{ab}	-3.60	16.45^{a}	14.26^{ab}	3.13
LSD (α=0.05)	15.21	11.65	-	8.42	9.46	-

Rknms – Reduction kernel number main spike, Rkwms – Reduction kernel weight main spike, RSkwms – Reduction Single kernel weight main spike

2.3.6. *Correlations*

Pearson's correlations were determined to understand the association between the physiological and yield components under high temperature stress (Table 7). The leaf temperature depression significantly correlated with both the flag leaf stomatal conductance and leaf cuticular wax content, although, stomatal conductance had a stronger correlation (r=0.33). Yield components, kernel number and kernel weight per main spike had a significant correlation with temperature depression and stomatal conductance. A significant negative correlation with the % reduction in yield components implies that cooler canopies will reduce the losses in yield components under high temperature stress. Flag leaf cuticular wax content was significantly associated with less reduction in kernel number and kernel weight of the main spike at 10DAP and 12DAP respectively. A significant negative correlation was observed between stomatal conductance and leaf cuticular wax content at 12DAP.

Table 7 Pearson's correlation analysis between the main spike yield components, flag leaf waxes, temperature depression and stomatal conductance under high temperature stress

	RKnms	RKwms	RSkrms	Wax (10D)	Wax (12D)	TD	St.Condt
RKnms	-	0.792**	0.250*	-0.241**	-0.054	-0.261*	-0.281*
RKwms		-	0.252*	-0.168	-0.179*	-0.211*	-0.237*
RSkrms				-0.0869	-0.127	-0.242*	-0.082
Wax (10D)				-	0.362**	0.172*	-0.01
Wax (12D)					-	0.232*	- 0.271*
TD						-	0.326**
St.Condt							-

* Significant at p=0.05, ** Significant at p=0.01
TD – leaf temperature depression, D – Days after pollination, St. Condt- Stomatal conductance
RKnms-Reduction kernel number main spike, RKwms- Reduction kernel weight main spike
RSkwms- Reduction single kernel weight main spike

2.4. Discussion

Variability for leaf wax content was found within the wheat cultivars at 10DAP. The amount of flag leaf cuticular wax content ranged between 0.5 to 2.91 mg dm $^{-2}$, which was similar to the reported leaf wax content previously by Uddin and Marshall (1988). The heat tolerant cultivars Halberd and Kauz had significantly higher wax content at 10DAP. The variation in the leaf cuticular wax content arises due to the differences in the biosynthesis and transport of waxes between the wheat cultivars. Studies in *Arabidopsis,* maize and barley have shown that several genes are involved in the biosynthesis and transport of leaf waxes and mutation in these genes alters the leaf wax

content (Samuels et al. 2008). Cuticular wax amounts are also regulated by developmental and environmental cues. In wheat Richard et al. (1986) reported that the amount of leaf cuticular wax content was highest after anthesis and gradually declined with grain filling. The wheat cultivars Halberd, Kauz, SeriM82, Ogallala, 7Cerros, Fang 60, Karl 92, Diebre and Australith had an increase in leaf cuticular wax content in response to high temperature stress at 12DAP. An increase in leaf waxiness has been reported in response to drought stress conditions in wheat (Johnson et al. 1983; Richards et al. 1986). Oat and rice cultivars grown in dry land conditions also have high leaf cuticular wax content (Uddin and Marshall 1988). The increased expression of ABC transporters and lipid transfer proteins (LTP) under high temperature stress conditions (Hays et al. 2007b) and their function as wax transporters may involve their role in increasing leaf wax content under high temperature stress. Interestingly the flag leaf cuticular wax content at 15 DAP, that is after stress, reduced in certain cultivars while it was further elevated in others.

Morphologically leaf epicuticular waxes were platelet shaped in all cultivars. The presence of octacosan-1-ol as a primary constituent of wheat waxes promotes the formation of platelet shape epicuticular wax crystals (Koch et al. 2006). The SEM results show that the lines with higher wax amounts had densely arranged wax platelets on their surface and higher average spectral reflectance in the photosynthetically active region (400-700nm). Studies in barley and wheat have shown an increase in 20% reflectance in the glaucous lines than the non-glaucous lines (Blum 1988, Febrero et al. 1998). Although, the thickness of the leaf cuticular wax layer was not estimated, the cultivar

Halberd had a thicker cuticular layer than the other cultivars in the study. A thicker cuticular layer will function as a barrier to water loss and a dense epicuticular layer will increase reflectance (Koch and Ensitak 2008).

Significant variation in leaf cooling was also observed between the cultivars. Leaf temperature depression is a complex phenomenon that has association with both leaf physiological and morphological parameters. Leaf temperature difference had a significant correlation with both leaf stomatal conductance and leaf cuticular waxes (Table 7). The wheat cultivars Halberd, Kauz, SeriM82 and 7 Cerros had with high leaf wax content and low stomatal conductance and significantly cooler leaves than other cultivars. Leaf temperature depression during high temperature stress has been associated as a direct function of evapo-transpiration (Reynolds et al. 1994). Amani et al. 1996 reported a positive correlation between canopy temperature depression and stomatal conductance in wheat. Johnson et al. (1983) observed that the stomatal conductance was higher in non-glaucous leaves than glaucous leaves under drought stress. Thus, in the wheat cultivars with low leaf cuticular wax content, stomatal conductance was a primary leaf cooling mechanism. The presence of higher amounts of leaf cuticular waxes significantly reduced leaf stomatal conductance in the wheat cultivars Halberd, Kauz, SeriM82 and 7Cerros. The presence of leaf cuticular waxes increased the reflectance of heat generating radiation and together with the stomatal conductance significantly reduced leaf temperatures in these cultivars.

The calculation of the reduction in yield components measured the response of the cultivars to stress. High temperature stress resulted in a reduction in both kernel

number and kernel weight of the main spike in the wheat cultivars. The heat tolerant cultivars Halberd, Kauz, SeriM82 and 7Cerros had significantly lower reduction in their kernel number and kernel weight. The lines Halberd, Kauz, SeriM82 and 7Cerros have been characterized previously as high yielding heat and drought germplasm. Yield reductions of 12-20% were observed in the heat susceptible lines Cutter, Australith, and Karl 92, Diebre and Fang 60. Both grain number and grain weight have been previously reported to be sensitive to high temperature stress in wheat with the number of grains per ear declining with increased temperatures at maturity (Ferris et al. 1998). The decline in grain weight is due to reduced starch accumulation as high temperatures inhibit photosynthesis (Blum 1986) and starch synthesis in the grain (Shipler and Blum 1990). Though the main effect of post-anthesis heat stress is in the reduction of kernel weight, other studies have also reported a reduction in kernel number resulting from short term high intensity heat stress shortly after pollination (Hays et al. 2007a; Tashiro and Wardlaw 1990). Kernel number and kernel weight both are primary components of yield. A significant positive correlation between the reduction in kernel number of the main spike and kernel weight of the main spike indicate that the primary reason for change in kernel weight under high temperature stress was due to kernel number and not single kernel weight.

The physiological traits leaf temperature depression, stomatal conductance and leaf cuticular waxes were associated with adaptation under high temperature stress. The reductions in yield components were negatively associated with the leaf temperature depression. That means wheat cultivars with cooler leaves had higher yields during high

temperature conditions. Halberd, Kauz, SeriM82 and 7Cerros had significantly cooler leaves than other cultivars and the observed yield reductions were lower. Leaf cooling was also significantly associated with stomatal conductance and leaf cuticular waxes. The reductions in yield components kernel number and kernel weight were associated negatively with stomatal conductance. Thus, increase in stomatal conductance under high temperature stress leads to reduction in leaf temperatures and maintains yield. Leaf cuticular waxes at 10DAP were associated with kernel number and at 12 DAP with kernel weight under heat stress conditions. Presence of leaf cuticular waxes serves as a protective function under high temperature stress. The cultivars with higher leaf wax content are likely to reflect the excess heat generating radiation thus reduce leaf temperatures and maintain yield.

Though evapo-transpiration has been identified as a primary mechanism for canopy temperature depression, based on the results of this study the presence leaf cuticular waxes in the plants also play a role in reducing leaf temperatures. The presence of thicker cuticular layers and dense network of leaf epicuticular waxes may increase reflectance, decrease canopy temperatures and maintain more effective control of water loss in plants and confer improved adaptation under high temperature stress.

CHAPTER III

QTL MAPPING OF LEAF CUTICULAR WAXES, CANOPY TEMPERATURE DEPRESSION AND YIELD COMPONENTS DURING REPRODUCTIVE STAGE HIGH TEMPERATURE STRESS

3.1. Introduction

Wheat is the most widely grown cereal in the world. Most of the wheat production is in the temperate, tropical and subtropical regions in the world. High temperature stress is one of the primary constraints to wheat growing in the tropical and subtropical areas. The average temperature in the U.S Great Plains during wheat flowering and grain filling is 28-30°C (Assad and Paulsen 2002). The hard winter wheat grown in this region shows susceptibility to heat stress in terms of its inability to maintain kernel number, kernel weight and grain filling duration (Hays et al. 2007b; Yang et al. 2002a). The intensity and duration of heat stress significantly impact yield. Both long term (Tashiro and Wardlaw et al. 1989; Yang et al. 2002a) and short-term heat stress (Hays et al. 2007a; Plaut et al. 2004; Tashiro and Wardlaw 1990) significantly influence both vegetative and reproductive processes in wheat when stress occurs in sensitive stages. However wheat is more sensitive during reproductive development in terms of sterility, grain abortion and grain filling duration. Post anthesis heat stress reduces both kernel numbers via abortion and kernel weight due to impaired maturation (Hays et al. 2007a; Plaut et al. 2004; Randall and Moss 1990; Tashiro and Wardlaw 1990). Short-term exposure to high temperature stress (1d at 35°C) reduces the activity of soluble starch

synthase and decreases starch deposition resulting in reduced grain weight (Hawker and Jenner 1993). A longer duration of heat stress significantly increases the rate of grain filling and reduces its duration (Spiertz 1974). Grain weight was also shown to reduce linearly with the increased duration of heat stress (Weigand and Cueller 1981). In the U.S. Great Plains moderate heat stress occurs on a yearly basis and extreme heat stress occurs routinely (Mason et al. 2010). Thus understanding heat tolerance under both short term and long-term high temperature stress is important. Heat tolerance is quantitative in nature and a few studies have reported quantitative trait loci (QTL) that improve heat tolerance (Yang 2002a, b; Mason et al. 2010) under short-term heat stress conditions.

Heat adaptive mechanisms such as modified increased photosynthetic rate and stomatal conductance and increased pubescence have been associated with yield under high temperature stress (Reynolds et al. 1994; Mason et al. 2010). A cooler canopy is beneficial for plants metabolic and physiological function, and can be an integrated outcome of these three adaptive mechanisms. We are interested in the role leaf cuticular waxes play in reducing leaf temperatures and increasing heat tolerance (Reynolds et al. 1994, Chapter II of this Dissertation). To date, there are no reports of genetic loci controlling leaf cuticular wax content in wheat. Defining QTL influencing the CTD and leaf cuticular waxes will provide an insight to its genetic regulation and their pleiotropic association with heat tolerance.

The objective of this study is to define QTL regulating leaf cuticular wax content and temperature depression and understand their association with reproductive stage heat tolerance during short-term and long-term heat stress.

3.2. Materials and methods

3.2.1. *Plant material*

A population of 121 recombinant inbred lines (RIL) derived from a cross between the heat tolerant cultivar ‘Halberd’ and the heat susceptible cultivar 'Karl92’ were characterized for leaf cuticular waxes and reproductive stage heat tolerance. The initial cross was developed in the greenhouse in 2003 and the lines were advanced by single seed descent to F5 after which they were bulked as F2:F5 (Mason et al. 2010). The F7 and F8 families were evaluated in the greenhouse in 2008 and 2009 respectively.

3.2.2. *Plant culture*

The recombinant inbred lines (RIL) and the parent cultivars were germinated in petri dishes and vernalized for six weeks at 4°C. The seedlings were planted in 12 x15 cm pots filled with a mixture of peat and sand in the ratio 1:3 peat. There were twelve replications of each line arranged in a completely randomized design in the greenhouse. Plants were fertilized with 5 grams of Osmocote™ and were supplemented with Peters™ (20:20:20) at the recommended rate once every two weeks starting from a two-leaf stage. Plants were grown at 20°C /18°C day / night temperatures with a 12hr photoperiod from 6 AM to 6 PM, under natural sunlight with 600 µmol m-2 s-1 PAR supplemental light. The first pollinated spike for each plant was tagged for day of pollination upon emergence of the anther from the spikelet.

3.2.3. *Stress treatment*

Two different heat stress regimes were followed, a short-term heat stress in 2008 and a long-term heat stress in 2009. A greenhouse set at 38°C/18°C day/night temperature was used for the heat treatment of RIL and parent cultivars in both years. Identical light conditions (supplemental light of 600 μmol m^{-2} s^{-1} PAR) were maintained in both the control and heat stress greenhouse. In 2008, at 10 DAP half of replications of each RIL were transferred to the heat stress greenhouse for a three day short-term heat stress while the second half were moved within the same greenhouse under control conditions. After heat treatment the plants were moved back to the control greenhouse and maintained until maturity.

In 2009 a long-term stress regime was followed where half of the replications of each RIL and parent cultivars were transferred to the heat stress greenhouse at 10 days after pollination (DAP) and maintained until maturity. The other half was moved within the control greenhouse maintained until maturity.

3.2.4. *Leaf cuticular waxes and spectral reflectance*

Leaf wax content was extracted and estimated from the flag leaf via a colorimetric technique described by Ebercon et al. (1977). The flag leaf samples were collected from RILs and parent cultivar at 10DAP. Flag leaf length and width were measured at 10DAP.

Six leaf discs of 1 cm in diameter were taken from each flag leaf sample and placed in a vial. The leaf wax was extracted with HPLC grade chloroform for 30 sec and vacuum dried. The extracted wax was oxidized with 300μl acidic potassium dichromate

($K_2Cr_2O_7$) in a water bath for 30 min. After cooling, 700ul of deionized water was added to each sample and allowed to develop color for an hour. The optical density of each sample was recorded at 590nm.

A standard curve was prepared following the similar procedure of wax extraction from the thirty random wheat flag leaves. The extracted leaf wax was weighed and re-dissolved in chloroform to prepare known concentration aliquots by the serial dilution technique. The resulting linear standard curve was used for calculating leaf wax quantity. Leaf reflectance measurements were taken only for the parent lines ‘‘Halberd’’ and ‘Karl92’ with a handheld spectrometer (Unispec-SC, PP Systems, Boston, MA).

3.2.5. *Temperature depression*

Flag leaf and main spike temperatures were taken between 11AM and 2PM with a handheld infrared thermometer (Model AG-42, Teletemperature Corp, Fullerton, CA). The measurements were taken at an angle of 45° to the horizontal. The temperature depression (TD) was estimated as the difference between the temperature of the heat/control greenhouse and the leaf/spike temperature.

3.2.6. *Yield component and agronomic trait measurement*

At maturity, the plants were harvested and threshed separately for primary spike and all other spikes were bulked. The yield components kernel number, kernel weight and single kernel weight were determined for the main spike. Only yield components from the main spike were used in data analysis to control for errors in the treatments associated with tillering and flowering of secondary tillers in the RILs (Mason et al.

2010; Yang et al. 2002a). Grain filling duration (GFD) was estimated as the date of pollination until 90% senescence of the main spike, days to flowering (Dtf) as the time from planting to pollination of the main spike, and days to maturity (Dtm) as the time from planting to senescence. A heat susceptibility index (HSI) for each individual RIL was calculated using the Fisher and Maurer equation (Fischer and Maurer 1978):

$$HSI = (1-Y_H/Y)/ (1-X_H/X)$$

Where, Y_H and Y are the phenotypic means for each line under heat stressed and control conditions, respectively, X_H and X are the phenotypic means for all lines under heat stressed and control conditions, respectively.

3.2.7. *Molecular mapping and QTL analysis*

A genetic linkage map for the 'Karl92' x ''Halberd'' population has already been created (Mason et al. 2010). The parent cultivars ''Halberd'' and 'Karl92' were screened with 623 wheat SSR markers. The polymorphic markers were used to screen the RIL population and generate a linkage map in JoinMap (Kyazma, B.V., Netherlands). A log of the likelihood score (LOD) of 3.0 and Kosambi's map function was used to establish linkage.

The phenotypic data for the QTL mapping include the leaf cuticular wax content, leaf and spike temperature depression, leaf length the leaf width and HSI for individual yield components for all RIL (Table 8).

QTL analyses used QTL Cartographer version 2.5 (WINQTL). Composite interval mapping (CIM) was used to determine the QTL positions and effects. A 1000 permutation test was used to determine the LOD threshold for each trait at a significance

level of P=0.05. A forward and backward regression method (p=0.05) with a 10cm window was used to identify QTL in CIM. The QTL were designated based on the nomenclature in the catalog for gene symbols for wheat (http://wheat.pw.usda.gov/ggpages/wgc/98/).

3.2.8. *Statistical analysis*

Statistical analysis was carried out using the GLM procedure (SAS v8.2, SAS Institute Inc., Cary, NC, USA). Significant differences between means of treatments were detected by considering treatment, genotypes and replication as fixed effect. The differences in mean HSI of the yield components under two heat treatment regimes were analyzed by T-test analysis in SAS v8.2. Pearson's correlations were also done using SAS v8.2.

Table 8 Phenotypic traits evaluated in the greenhouse for ''Halberd'' x 'Karl92' population in 2008 and 2009

Trait	QTL symbol	Method of Measurement
Kernel number of main spike	knm	Number of kernels of main spike at maturity
Kernel weight of main spike (g)	kwm	Yield of main spike at maturity
Single kernel weight of main spike (g)	skm	Kernel weight of main spike / kernel number of main spike
HSI	HSI or H	Heat susceptibility index calculated for each yield component
Days to flowering (days)	Dtf	Days from planting to flowering of main spike
Days to maturity (days)	Dtm	Days from planting to 90% senescence of the main spike
Grain-filling duration (days)	Gfd	Days from flowering to 90% senescence of main spike
Flag leaf length (cm)	Fll	Length of flag leaf from base of leaf to tip
Flag leaf width (cm)	Flw	Width of the widest section of the flag leaf
Temperature depression (°C)	Td	Air temperature minus organ temperature, measured using a handheld infrared thermometer
Leaf cuticular waxes (mg / dm^2)	Wax	Flag leaf cuticular wax content estimated by colorimetric technique

3.3. Results

3.3.1. *Effects of heat stress on physiological components in the parent cultivars*

The parent cultivar ‘‘Halberd’’ had significantly higher leaf cuticular wax content at 10 DAP in the both the years 2008 and 2009 (Table 9). The flag leaf spectral reflectance measurements taken on the parent cultivars indicate that ‘Halberd’ had a higher average reflectance in the visible and the near infrared region than the heat susceptible parent ‘Karl92’ (Fig. 8). There was significant variability for cuticular leaf wax content in the recombinant inbred line population (RIL) in both the years. The leaf cuticular wax content ranged from 3.08 to 1.23 mg/dm^{-2} in 2008 and 3.37 to 1.02 mg/dm^{-2} in 2009 (Fig. 8).

Leaf and spike temperatures were recorded for the parent cultivars ‘Halberd’ and ‘Karl92’ and the RIL population under high temperature stress. Both the cultivars and the RIL population responded to high temperature stress by lowering their leaf and spike temperatures relative to the stress temperature of 38°C. The mean of leaf and spike temperature depressions in 2008 and 2009 are presented in Table 9. ‘Halberd’ had significantly cooler leaf temperatures of 5-7 °C in both the years. Compared to ‘Karl92’, spike temperatures were significantly lower in ‘Halberd’ in both the years. The heat tolerant line ‘Halberd’ maintained a cooler leaf and spike temperatures both during the short-term heat stress and long-term heat stress conditions. Leaf temperature depression in the recombinant inbred lines ranged from 1.7 to 7°C in 2008 and 2 -7°C in 2009 (Fig. 9). The flag leaf length and width of the parent cultivars and RIL were measured at 10DAP. ‘Halberd’ had longer and wider leaves than ‘Karl92’ in both years. Significant

genotypic variation in leaf flag leaf length and width was also observed in the RIL population in 2008 and 2009.

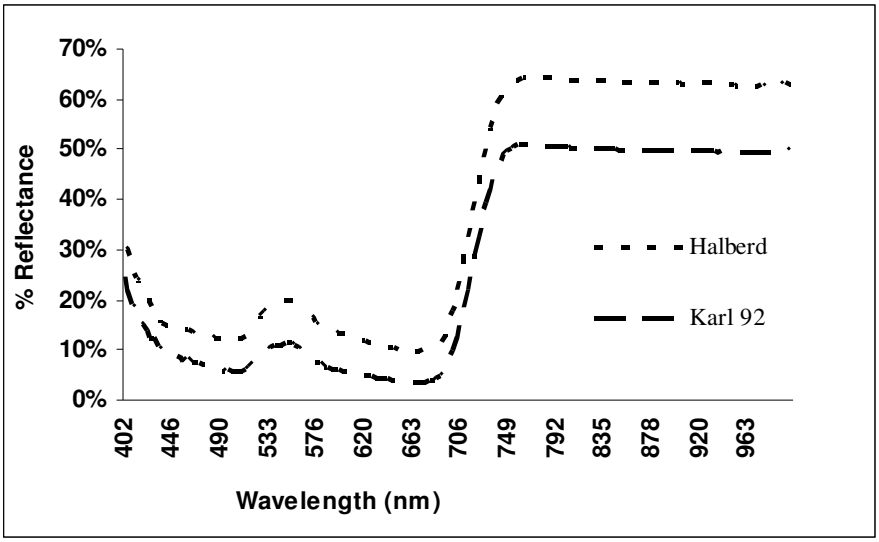


Fig. 8 Leaf reflectance spectra of the flag leaf of parent lines 'Halberd' and 'Karl92' at 10DAP

Table 9 Data for physiological components in parent cultivars (Mean ± SE) and recombinant inbred lines (mean ± SD) in greenhouse 2008 and 2009

	Halberd		Karl92		Recombinant Inbred Lines	
Trait	**2008**	**2009**	**2008**	**2009**	**2008**	**2009**
Temperature depression leaf (°C)	6.8 ± 1.3	5.6 ± 1.41	6.2 ± 1.3	4.3 ± 1.03	4.65* ± 1.21	3.68** ± 1.56
Temperature depression spike (°C)	4.5 ± 1.0	4.5 ± 1.16	3.7 ± 1.0	2.9 ± 0.6	1.82* ± 1.33	2.4** ± 1.11
Flag Leaf wax at 10DAP (mg/dm^{-2})	2.59 ± 0.17	2.63 ± 0013	1.72 ± 0.31	1.68 ± 0.031	2.35** ± 0.03	1.77* ± 0.05
Flag leaf length (cm)	28.6 ± 1.4	25.2 ± 1.4	23.5 ± 1.8	20.2 ± 1.8	23.4** ± 6.9	22.7** ± 3.5
Flag leaf width (cm)	1.40 ± 0.02	1.37 ± 0.04	1.28 ± 0.04	1.18 ± 0.08	1.24* ± 0.25	1.11 ± 0.64

** Significant at p =0 .01, * Significant at p = 0.05 for the RIL population in 2008 and 2009

3.3.2. *Effects of heat stress on yield components in the parent cultivars*

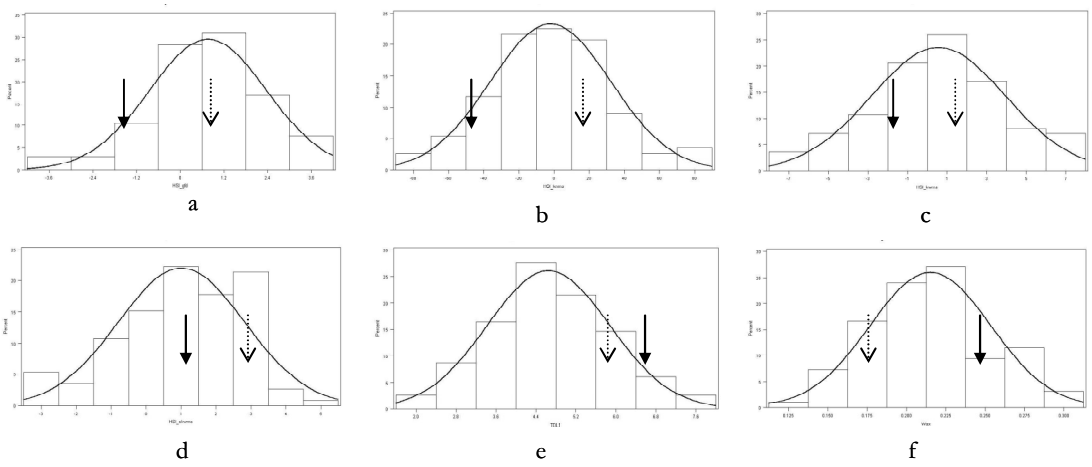
High temperature stress resulted in a reduction of yield components in the main spike. During a 3 day short-term high temperature stress at 38°C, the heat tolerant cultivar ‘‘Halberd’’ exhibited no significant reductions in kernel number, kernel weight or single kernel weight of the main spike (Table 10). However, during short-term temperature stress ‘Karl92’ had a reduction in kernel number and kernel weight (Table 10). In 2009, the long-term heat stress treatment resulted in a significant reduction in kernel weight but not kernel number in ‘Halberd’. However, during long-term stress ‘Karl92’ had significant reductions in both kernel number and kernel weight. No significant reduction in single kernel weight was observed in the parent cultivars during either short term or long-term heat stress conditions. The RIL population had significant treatment effects

for kernel weight and single kernel weight during short-term heat stress in 2008 (Table 10). During the long-term heat stress conditions in 2009 the RIL population had significant reductions all the main spike yield components.

The days to flowering were significantly different in the two parent cultivars and within the RIL population. During long-term heat stress the grain filling duration (GFD) reduced significantly in ‘‘Halberd’’. In ‘Karl92’ GFD was reduced during heat treatment in both 2008 and 2009 (Table 10). There were significant reductions in GFD during both short-term and long-term heat stress conditions in the RIL population. Differences in days to maturity were a result of significant differences in the flowering time and grain filling duration during the two heat stress conditions.

Based on the treatment differences observed in short-term and long-term heat stress conditions the heat susceptibility index (HSI) was estimated for the main spike yield components of the RIL population. The higher positive values of HSI indicate susceptibility. The impact of short-term heat stress and long-term heat stress on the main spike yield components of the RIL population can be compared using on the heat susceptibility index (Table 10). Significantly high positive values of HSI were observed for kernel number and kernel weight during long-term heat stress conditions (Table 10). The grain filling duration also had significantly high HSI during long-term heat stress. Long-term heat stress resulted in a significant reduction in the main spike kernel number, kernel weight and the GFD. The HSI of the main spike yield components were normally distributed (Fig. 9). The heat tolerant cultivar ‘‘Halberd’’ had lower values of HSI for the main spike yield components (Fig. 9).

Yield components and physiological traits in 2008



Yield components and physiological traits in 2009

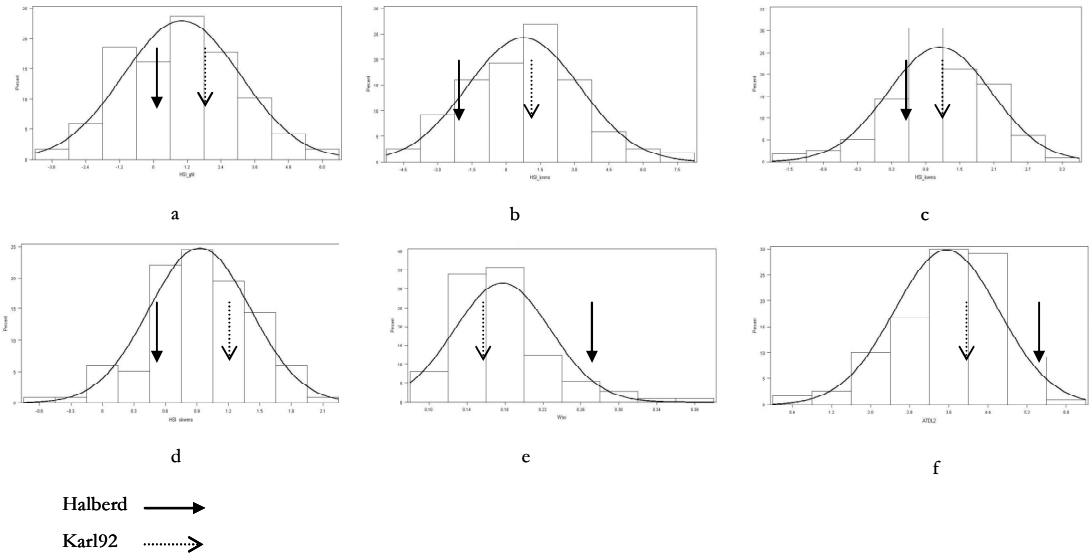


Fig. 9 Normality curves for yield and physiological traits in 2008 and 2009. The traits included in 2008 are a) HSI kernel number b) HSI kernel weight c) HSI single kernel weight d) leaf cuticular wax content e) leaf temperature depression f) spike temperature depression. The traits in 2009 are a) HSI kernel number b) HSI kernel weight c) HSI single kernel weight d) leaf cuticular wax content e) leaf temperature depression f) spike temperature depression

Table 10 Data for yield components in parent lines 'Halberd' and 'Karl92' (Mean ± SE) and recombinant inbred lines (mean ± SD) in greenhouse in 2008 and 2009 during control and heat-treated conditions

		''Halberd''		'Karl92'		Recombinant Inbred Lines		
Trait	**Year**	**Control**	**Treated**	**Control**	**Treated**	**Control**	**Treated**	**HSI[1]**
Kernel number/main spike	2008	46.1 ± 1.2	42.4 ± 1.9^{ns}	34.9 ± 2.7	21.7 ± 2.6*	30.8 ± 11.9	31.2 ± 12.6^{ns}	-1.54$^{\#}$
Kernel number/main spike	2009	38.4 ± 1.8	32.6 ± 1.3^{ns}	19.1 ± 1.64	17.8 ± 1.9	28.7 ± 7.9	26.5 ± 6.7**	1.23
Kernel weight/main spike (g)	2008	1.71 ± 0.1	1.73 ± 0.1^{ns}	1.24 ± 0.10	0.78 ± 0.10*	1.07 ± 0.5	1.02 ± 0.4*	0.57$^{\#}$
Kernel weight/main spike (g)	2009	1.41 ± 0.3	1.18 ± 0.2 **	0.6251 ± 0.10	0.5541 ± 0.1**	1.06 ± 0.5	0.8 ± 0.4**	1.49
Single kernel weight/main spike (g)	2008	0.037 ± 0.001	0.041 ± 0.002^{ns}	0.036 ± 0.001	0.036 ± 0.001^{ns}	0.035 ± 0.008	0.033 ± 0.007**	1.01
Single kernel weight/main spike (g)	2009	0.037 ± 0.005	0.036 ± 0.002^{ns}	0.032 ± .004	0.0030 ± 0.003	0.037 ± 0.02	0.030 ± 0.015	0.94
Grain-filling duration (days)	2008	39.0 ± 1.4	37.7 ± 0.7^{ns}	35.6 ± 0.9	33.6 ± 0.7*	35.9 ± 3.2	34.6 ± 3.4**	1.24$^{\#}$
Grain-filling duration (days)	2009	34.0 ± 1.4	29 ± 0.7**	35.6 ± 0.9	23.6 ± .12**	33.7 ± 1.23	25.6 ± 1.72**	1.64
Days to maturity (days)	2008	95.7 ± 0.3	97.0 ± 1.1^{ns}	82.8 ± 1.4	81.0 ± 0.9^{ns}	90.6 ± 9.1	89.7 ± 9.6^{ns}	0.82
Days to maturity (days)	2009	91.7 ± 0.3	82.4 ± 1.1^{ns}	82.8 ± 1.4	74.0 ± .8**	90.6 ± 9.1	81.7 ± 9.6^{ns}	1.04
Days to flowering (days)	2008	57.0 ± 1.0	59.0 ± 1.5^{ns}	47.1 ± 0.3	47.4 ± 0.3^{ns}	56.4 ± 10.8	56.2 ± 11.3^{ns}	-
Days to flowering (days)	2009	57.0 ± 1.0	56.0 ± 1.5^{ns}	47.1 ± 0.3	48.4 ± 0.3^{ns}	56.4 ± 10.8	55.2 ± 11.3^{ns}	-

** Significant at p =0 .01, * Significant at p = 0.05 between control and heat-treated plants of parental and recombinant inbred lines with treatment as a fixed effect

[1] Average HSI calculated across all RIL

$^{\#}$Significant differences in the HSI index of the recombinant inbred line population in 2008 and 2009 at p = 0.05

3.3.3. *Correlations of the physiological and phenotypic traits during short-term heat stress conditions.*

The main spike HSI yield components were correlated with flag leaf cuticular wax content and the leaf temperature depression during short term heat stress in 2008 (Table 11). The flag leaf cuticular waxes had a significant negative correlation with the HSI of kernel weight HSI. A negative correlation indicates that leaf cuticular waxes were associated with lower values of HSI. Lower HSI values are associated with tolerance to high temperatures. The leaf cuticular waxes had a weak correlation with leaf temperature depression (Tdl) on day 2. The leaf temperature depressions (Tdl) recorded on the day1 and day 2 of the short-term heat stress was significantly associated with the HSI of kernel number, kernel weight and single kernel weight. The spike temperature depression (Tds) recorded on day 2 was correlated with the kernel number and kernel weight HSI. The main spike HSI for yield components were moderately to strongly correlate with each other except for HSI of kernel number and single kernel weight that had no correlations.

Table 11 Pearson's correlation coefficient values for HSI of yield components, leaf cuticular wax content and temperature depression in 2008

	Tdl1	Tds1	Tdl2	Tds2	Wax	HKn	Hkw	HSkw	Fll	Flw
Td leaf Day1 (Tdl1)	-	0.29**	0.37**	0.04	0.109	-0.12	-0.25*	-0.28**	0.02	0.05
Td spike day1 (Tds1)		-	0.08	0.50**	0.05	-0.02	-0.00	0.00	0.20*	0.09
Td leaf day 2 (Tdl2)			-	0.16	0.12*	-0.17*	-0.19*	-0.18*	-0.02	-0.03
Td spike day 2 (Tds2)				-	0.079	-0.22*	-0.24*	-0.07	-0.09	-0.08
Wax (10DAP)					-	-0.15	-0.16*	-0.07	0.015	0.043
HSI kernel number(HKn)						-	0.88**	0.059	-0.12	0.04
HSI kernel weight (Hkw)							-	0.32**	-0.14	0.069
HSI single kernel weight (HSkw)								-	-0.07	0.10
Flag leaf length (Fll)									-	0.21*
Flag leaf width (Flw)										-

Td - temperature depression, HSI- heat susceptibility index
* Significance at p=0.05 level ** Significance at p=0.01

3.3.4. *Correlations of the physiological and phenotypic traits during long-term heat stress condition*

The results from the correlation analysis between the physiological and phenotypic traits during long-term heat stress in 2009 were summarized in Table 12. The main spike HSI for yield components had no correlations with the flag leaf cuticular wax content and spike temperature depression. Leaf temperature depression on day 1was moderately associated with HSI for kernel weight and on day 2 with HSI for kernel weight and kernel number. A negative correlation is favorable. Thus keeping the leaves cooler under high temperature stress was associated with reduced losses in kernel weight under high temperatures. Leaf temperature depression on day 1 of high temperature stress had weak

correlation with leaf cuticular waxes at 10DAP. The HSI of kernel number was strongly correlated with HSI of kernel weight and single kernel weight.

Table 12 Pearson's correlation coefficient values for HSI of yield components, flag leaf cuticular wax and temperature depression in 2009

	Tdl1	Tds1	Tdl2	Tds2	Wax	HKn	Hkw	HSkw	Fll	Flw
Td leaf Day1 (Tdl1)	-	0.58**	0.29*	0.28*	0.14*	-0.112	-0.27*	-0.05	0.09	0.07
Td spike day1 (Tds1)		-	0.53*	0.56**	0.07	0.06	0.033	-0.11	0.08	0.24
Td leaf day 2 (Tdl2)			-	0.65**	0.09	-0.25*	-0.27*	-0.06	0.018	0.023
Td spike day 2 (Tds2)				-	0.04	0.08	0.054	-0.22	0.035	0.08
Wax (10DAP)					-	-0.11	-0.13	-0.07	0.105	0.04
HSI kernel number (HKn)						-	0.95**	-0.32*	0.13	0.32*
HSI kernel weight (Hkw)							-	-0.10	0.15	0.25
HSI single kernel weight (HSkw)								-	-0.003	-0.08
Flag leaf length (Fll)									-	0.19
Flag leaf width (Flw)										-

Td- temperature depression, HSI - heat susceptibility index
* Significance at p=0.05, ** Significance at p= 0.01

3.3.5. *Linkage mapping and QTL analysis*

The genetic linkage map was constructed with 189 SSR markers and one phenotypic (B1/awns) markers using JoinMap software (Mason et al. 2010). Markers were unevenly distributed with some significant gaps within the linkage groups present on chromosome 3D, 4A, 4D and 6D. The marker order was highly conserved with respect to previous wheat genetic maps and consensus SSR maps (Somers et al. 2004).

3.3.6. *Composite interval mapping in 2008 during short-term high temperature stress*

The main spike HSI for yield components were mapped to identify QTL associated with yield stability under high temperature stress. The flag leaf cuticular wax content and leaf and spike temperature depression were also mapped to detect QTL associated with individual traits and to identify pleiotropy that may influence reproductive stage heat tolerance.

In 2008 during short-term heat stress conditions we identified 14 QTL associated with the main spike HSI yield components (Table 13). A single QTL on 5B was identified for the HSI of kernel number. The susceptible line 'Karl92' contributed the favorable allele and the QTL explained 13% of the phenotypic variation. Two QTL for kernel weight HSI were identified on 1B and 5A. The QTL *QHkwm.tam-1B* explained 10% of the phenotypic variation and the favorable allele was contributed by heat tolerant line ''Halberd''. The other two QTL for HSI of kernel weight were located 20cM apart on the distal region of the long arm of the chromosome 5A. The favorable allele for the QTL *QHkwm.tam-5A1* was contributed by 'Halberd' and it accounted for 12% of the phenotypic variation. The other QTL *QHkwm.tam-5A2* was contributed by 'Karl92' and explained 11% of the variation. Ten QTL were identified for HSI of single kernel weight that explained 4.5% to 19.3% of the phenotypic variation. 'Halberd' contributed favorable alleles in seven of the ten QTL identified. Putative QTL were identified for days to flowering in chromosomes 2D and 3A. No significant QTL were identified for grain filling duration.

Table 13 QTL detected in the 'Halberd' x 'Karl92' mapping population (n=121) in the greenhouse 2008

QTL (LOD threshold[a])	Marker	LOD	R^2	Additive[b]	Positive allele
HSI_Kernel number of main spike (3.03)					
QHknm.tam-5B	gwm408	3.05	0.134	1.28	Karl92
HSI_Kernel weight of main spike (3.25)					
QHkwm.tam-1B	gwm153	3.93	0.101	-1.19	Halberd
QHkwm.tam-5A.1	gwm179	3.95	0.122	-1.49	Halberd
QHkwm.tam-5A.2	gwm291	3.81	0.114	1.38	Karl92
HSI_Single kernel weight main spike (3.15)					
QHskm.tam-2D.1	gwm261	11.77	0.193	0.92	Karl92
QHskm.tam-2D.2	cfd56	3.61	0.052	-0.49	Halberd
QHskm.tam-3B	barc229	3.17	0.045	-0.41	Halberd
QHskm.tam-4A.1	wmc707	5.50	0.096	-0.79	Halberd
QHskm.tam-4A.2	wmc313	7.55	0.123	0.87	Karl92
QHskm.tam-5A	gwm443	4.04	0.058	0.47	Karl92
QHskm.tam-5B	wmc73	4.08	0.062	-0.50	Halberd
QHskm.tam-6D	cfd42	6.01	0.147	-0.75	Halberd
QHskm.tam-7A	wmc603	4.27	0.093	-0.76	Halberd
QHskm.tam-7B	wmc182	3.79	0.055	-0.47	Halberd
Days to flowering (3.05)					
QDtf.tam-2D.1c	gwm484	3.80	0.152	4.02	Halberd
QDtf.tam-2D.2c	wmc41.1	4.15	0.095	-3.34	Karl92
QDtf.tam-3Ac	gwm369	3.87	0.096	-3.22	Karl92
Days to maturity (5.1)					
QMat.tam-2D	cfd56	5.92	0.235	4.05	Halberd
Flag leaf cuticular waxes (3.12)					
QWax.tam-1B	wmc469	3.22	0.096	-0.139	Karl92
QWax.tam-5A	wmc713	3.81	0.121	0.016	Halberd
Temperature depression of flag leaf day (2.93)					
QTdl.tam-3Bc	barc84	2.75	0.093	0.44	Halberd
QTdl.tam-5A.1	gwm154	3.17	0.113	-0.60	Karl92
QTdl.tam-5A.2	gwm179	3.18	0.093	0.46	Halberd
Temperature depression of main spike day (2.89)					
QTds.tam-2D.1	gwm261	5.46	0.110	-0.51	Karl92
QTds.tam-2D.2	cfd56	4.07	0.122	0.55	Halberd
QTds.tam-5A.1	gwm126	6.74	0.146	0.68	Halberd
QTds.tam-5A.2	gwm595	4.61	0.088	-0.56	Karl92
QTds.tam-6D	cfd42	9.71	0.321	0.81	Halberd
Flag leaf length (3.35)					
QFll.tam-1B	wmc156	3.08	0.038	1.31	Halberd
QFll.tam-2D	gwm484	18.37	0.328	3.85	Halberd

Table 13 (continued)

QTL (LOD threshold[a])	Marker	LOD	R^2	Additive[b]	Positive allele
Flag leaf width (3.37)					
QFlw.tam-2D	gwm484	9.84	0.177	0.07	Halberd

[a] LOD thresholds were estimated in QTL Cartographer v2.0 using 1000 permutation

[b] Additive effect of allele substitution

[c] Putative QTL

Two QTL were identified for flag leaf cuticular waxes. The favorable allele for QTL *QWax.tam-1B* located on chromosome 1B was contributed by 'Karl92' and explained 9% of the phenotypic variation. The other QTL *QWax.tam-5A* located on chromosome 5A was contributed by 'Halberd' and explained 12% of the variation. Of the three QTL identified for flag leaf temperature depression one was a putative QTL located on 3B contributed by 'Halberd'. The other two QTL for leaf temperature depression were contributed by 'Halberd' and 'Karl92' respectively and located on chromosome 5A each explaining 9% of the phenotypic variation. A single QTL for spike temperature depression was located on 6D while two each were located on 2D and 5A. The QTL *QTds.tam-6D* on chromosome 6D explained 32% of the variation and was contributed by 'Halberd'. The other QTL for spike temperature depression explained 8% to 14% of the phenotypic variation. The QTL for flag leaf length and width were co-localized on chromosome 2D. The favorable allele for the QTL was contributed by 'Halberd' for both the traits.

3.3.7. *Composite interval mapping in 2009 during long-term high temperature stress*

A summary of QTL detected during long-term high temperature stress is presented in Table 14. During long-term heat stress conditions six QTL were identified that were associated with HSI for the main spike yield components (Table 14). One QTL was identified for HSI of kernel number on chromosome 7D. The positive allele for the QTL *QHknm.tam-7D* was contributed by 'Halberd' and explained 9% of the phenotypic variation. The QTL for kernel weight HSI was identified on 1B, 1D and 5B. The favorable allele for all the three QTL was contributed by 'Halberd' and explained 9%-11% of the phenotypic variation. The QTL *QHskm.tam-1B* and *QHskm.tam-1D* were associated with single kernel weight HSI and explained 19% and 22% of the phenotypic variation respectively. 'Karl92' contributed the favorable allele on QTL *QHskm.tam-1B* and 'Halberd' on QTL *QHskm.tam-1D*.

No significant QTL were identified for GFD. Three QTL were identified for days to flowering and one QTL for days to maturity. The QTL for days to flowering on 2D co-localized with the single QTL identified for days to maturity.

QTL for flag leaf cuticular waxes were identified on chromosome 1B, 3D and 5A. The QTL *QWax.tam-1B* was contributed by 'Karl92' and explained 8% of the phenotypic variation. The favorable allele for the QTL *QWax.tam-3D* and *QWax.tam-5A* were contributed by 'Halberd' and each explained 9% -11% of the phenotypic variation. The flag leaf wax QTL *QWax.tam-5A* was also identified in the greenhouse in 2008 (Table 13).

Four QTL were identified for flag leaf temperature depression. The QTL on 2D was contributed by 'Karl92' and explained 17% of the phenotypic variation. 'Halberd' contributed the favorable allele in the QTL *QTdl.tam-3B* that explained 8% of the phenotypic variation. The two QTL on 5A were previously identified in 2008 and are present on the distal end of the long arm of chromosome 5. Of the two QTL for temperature depression *QTdl.tam-5A.1* was contributed by 'Karl92' the other QTL *QTdl.tam-5A.2* was contributed by 'Halberd' and each explained 16% and 18% of the phenotypic variation respectively. Three QTL identified for spike temperature depression were located on chromosome 3B, 5A and 5B. 'Halberd' contributed the favorable alleles in QTL *QTds.tam-3B* and *QTds.tam-5A* and 'Karl92' contributed favorable alleles to the QTL *QTds.tam-5B*.

The QTL explained 8% -14% of the phenotypic variation. The favorable allele for both the QTL identified for flag leaf length was contributed by 'Karl92' and explained 8-12% of the phenotypic variation. One QTL for flag leaf length on 6D co-localized with *QFlw.tam-6D*, a QTL for flag leaf width.

Table 14 QTL detected in the 'Halberd' x 'Karl92' mapping population (n=121) in the greenhouse, 2009

QTL (LOD threshold[a])	Marker	LOD	R^2	Additive[b]	Positive allele
HSI_Kernel number of main spike (3.03)					
QHknm.tam-7D	gwm635.2	3.18	0.096	-1.07	Halberd
HSI_Kernel weight of main spike (3.05)					
QHkwm.tam-1B	wmc419	3.11	0.096	-0.34	Halberd
QHkwm.tam-1D	cfd15	3.21	0.083	-0.485	Halberd
QHkwm.tam-5B	gwm544	3.83	0.112	-0.046	Halberd
HSI_Single kernel weight main spike (3.01)					
QHskm.tam-1B	barc240	3.59	0.097	0.199	Karl92
QHskm.tam-1D	barc148	3.08	0.106	-0.225	Halberd
Days to flowering (3.0)					
QDtf.tam-2A	gwm645	3.25	0.078	-0.0723	Halberd
QDtf.tam-2D	gwm484	3.02	0.184	1.099	Karl92
QDtf.tam-3A	gwm5	3.20	0.886	0.0657	Karl92
Days to maturity (3.1)					
QMat.tam-2D[c]	gwm261	2.60	0.071	-0.1826	Halberd
Flag leaf cuticular waxes (3.17)					
QWax.tam-1B	wmc156	3.42	0.088	-0.016	Karl92
QWax.tam-3D	gwm191	3.78	0.118	0.018	Halberd
QWax.tam-5A	gwm205	3.31	0.096	0.0165	Halberd
Temperature depression of flag leaf day (3.02)					
QTdl.tam-2D	cfd56	4.48	0.17	-0.45	Karl92
QTdl.tam-3B	wmc418	3.03	0.085	0.37	Halberd
QTdl.tam-5A.1	barc186	3.9	0.165	-0.58	Karl92
QTdl.tam-5A.2	barc141	3.95	0.188	0.68	Halberd
Temperature depression of main spike day (3.19)					
QTds.tam-3B	wmc418	5.17	0.14	0.39	Halberd
QTds.tam-5A	gwm293	3.19	0.084	0.36	Halberd
QTds.tam-5B	gwm160	3.64	0.088	-0.36	Karl92'
Flag leaf length (3.35)					
QFll.tam-1B	gwm268	3.32	0.078	-1.07	Karl92
QFll.tam-6D	gwm620	4.7	0.127	-1.347	Karl92
Flag leaf width (3.10)					
QFlw.tam-2D[c]	wmc144	2.91	0.084	0.178	Halberd
QFlw.tam-6D	cfd42	3.17	0.085	-0.131	Karl92

[a] LOD thresholds were estimated in QTL Cartographer v2.0 using 1000 permutation
[b] Additive effect of allele substitution
[c] Putative QTL

3.4. Discussion

3.4.1. *Effect of heat stress on main spike yield components*

Reductions in main spike yield components were observed during both short-term and long-term heat stress conditions in the heat susceptible parent 'Karl92' and the RIL population. 'Halberd' had non-significant changes in yield components during short-term heat stress but had significant reductions in kernel weight per main spike during long-term heat stress. Previous studies with these cultivars have shown heat tolerance in 'Halberd' and susceptibility in 'Karl92' during short-term heat stress (Mason et al. 2010, Hays et al. 2007a, Yang et al. 2002a). A study on the acquisition and maintenance of thermotolerance has found that 'Halberd' was tolerant to short periods of high temperature stress but was unable to maintain the level of protection under longer duration of stress (Blumenthal et al. 1990). A comparison of the HSI of the yield components in the RIL population illustrates the impact of the duration high temperature stress (Table 10). The HSI for the main spike yield components were comparatively higher for the RIL population during long-term heat stress. Compared to the short-term heat stress, the HSI for grain filling duration of the RIL population was higher during long-term stress. Thus increasing the duration of high temperature stress shortened the duration of grain filling in RIL population.

3.4.2. *Effect of heat stress on physiological traits*

Flag leaf and spike temperatures were lower than the stress temperature (38°C). 'Halberd' had significantly lower leaf and spike temperature than 'Karl92'. The RIL population was normally distributed with some lines showing transgressive segregation

for the temperature depression (Td) of leaf and spike. The flag leaf Td was correlated with HSI for yield components during both short-term and long-term heat stress conditions. Spike Td was correlated with HSI for main spike yield components during short-term heat stress, not during long-term heat stress. Canopy temperature depression that includes both leaf and spike temperature depression has previously been reported to associate with yield under high temperature stress (Mason et al. 2010; Ayeneh et al. 2002; Reynolds et al. 2000). The results suggest that cooling of leaf and spike may be important physiological adaptive mechanism under short periods of high temperature stress.

Leaf cuticular waxes were estimated on 10DAP only. Previous study described in Chapter II of this dissertation shows the presence of higher amounts of leaf cuticular waxes may serve a function to reduce leaf temperature and maintain yield under high temperature stress conditions. The heat tolerant cultivar 'Halberd' had significantly higher flag leaf wax content than the heat susceptible cultivar 'Karl92'. The flag leaf cuticular wax content in the RIL population was normally distributed with some lines showing transgressive segregation. During short-term heat stress leaf cuticular waxes were correlated with the leaf temperature depression and HSI for kernel weight. During long-term heat stress, leaf cuticular waxes were weakly correlated with leaf Td but had no significant correlations with the HSI of the yield components. The leaf cuticular waxes may play a role in the initial adaptation to the high temperature stress through a reduction of leaf temperatures and contributing to yield stability but may not serve a role during long-term acclimation process. Plants may redirect their resources to grain filling

under long duration high temperature stress and less to leaf wax production. The rate of grain filling has been reported to increase with duration of high temperature stress (Spiertz 1974).

3.4.3. *Co-localization of HSI, TD and Wax QTL during short-term stress*

The QTL analysis identified loci associated with the phenotypic and physiological traits during short-term high temperature stress (Table 13). A total of 14 QTL were identified for HSI of main spike yield components. Two of the three favorable alleles for the HSI of kernel weight and seven of the ten QTL for the HSI of single kernel weight were from 'Halberd'. Individual QTL explained 4.5% to 19.3% of the variance in the main spike yield traits. Overall, the QTL explained 13% and 34% the phenotypic variance in the HSI of the main spike kernel number and kernel weight respectively.

The QTL for flag leaf cuticular waxes were identified on 1B and 5A explaining a total of 20% variation in the cuticular waxes. The *QWax.tam-1B* on the short arm of chromosome 1 and is located near to a previously identified spike non-glaucousness loci (Dubcovsky et al. 1997). The QTL for HSI of kernel weight, Td of leaf and spike and wax were co-localized at loci on the chromosome 5AL. *QHkwm.tam-5A.1, QTdl.tam-5A.2, QTds.tam-5A.1* and *QWax.tam-5A* explained 9.3%, 14.6%, 12.2% and 12.6% of the variation in the respective traits. 'Halberd' contributed favorable allele at all the loci. While the vernalization gene VrnA1 resides on 5AL region, no significant loci for flowering time were detected on 5AL in this study. QTL for HSI and temperature depression were co-localized at seven loci including the one on 5A. Two spike temperature depression loci co-localized with QTL for kernel weight HSI and single

kernel weight HSI. The *QHkwm.tam-5A.2* and *QTds.tam-5A.2* co-localized on short arm of chromosome 5 and 'Karl92' contributed the favorable allele. The spike Td QTL *QTds.tam-6D* co-localized with the *QHskm.tam-6D*, a QTL for single kernel weight HSI. This was a large effect QTL and explained 32.1% of the variation in spike Td and 14.5% of variation in single kernel weight. The flowering time loci were identified on 2D, which localized on a previously identified photoperiod loci, Ppd-D1, one of the major photoperiod loci in wheat. The QTL for HSI of main spike yield components, Td of leaf and spike were co-localized with the two flowering time loci *Qdtf.tam-2D.1* and *Qdtf.tam-2D.2*. While some QTL did co-localize with the flowering time QTL, the other loci were independent of flowering time.

3.4.4. *Co-localization of HSI, TD and Wax QTL during long-term stress*

During long-term heat stress we identified 7 QTL for HSI of main spike yield components (Table 14). Though the individual QTL identified had small effects, overall they explained 9%, 28% and 20% of the variation in HSI for kernel number, kernel weight and single kernel weight of the main spike respectively. The three QTL identified for leaf cuticular wax explained a total of 27% of the variation. The QTL *QWax.tam-1B* co-localized with *QHskm.tam-1B*, a QTL for single kernel weight HSI. 'Karl92' contributed the favorable allele in both the loci. Thus the wax QTL on 1B will reduce leaf cuticular wax content. The other wax QTL on the long arm of chromosome 5A co-localized with the leaf and spike Td loci. The favorable alleles in all the three co-localized QTL were contributed by 'Halberd'. Leaf and Spike Td QTL *QTdl.tam-3B* and *QTds.tam-3B* co-localized on chromosome 3B and had favorable alleles from 'Halberd'.

The co-localization of the wax QTL with the QTL for single kernel weight HSI and leaf and spike temperature depression suggests that plant adaptive traits may influence heat tolerance.

The major photoperiod gene Ppd-1 in wheat in located on 2D. A significant flowering time QTL was identified near the Ppd-1 region. The *Qdtf.tam-2D* also co-localized with leaf and spike Td QTL. None of the QTL for HSI of the yield components or leaf cuticular waxes were co-localized with flowering time and thus the QTL identified for these traits were independent of the flowering time.

The mean allele effects of the markers associated leaf cuticular wax content QTL *QWax.tam-5A* and *QWax.tam-1B* and leaf temperature depression QTL *QTdl.tam-3b* in 2008 and 2009 are presented in Fig. 10. The three loci had positive allele from 'Halberd' and co-localized with HSI of main spike yield components. Though significant differences in allele effects were not observed at all loci, the 'Halberd' allele was beneficial. The RIL with the 'Halberd' allele had lower HSI for kernel weight per main spike, higher leaf cuticular wax content and leaf temperature depression.

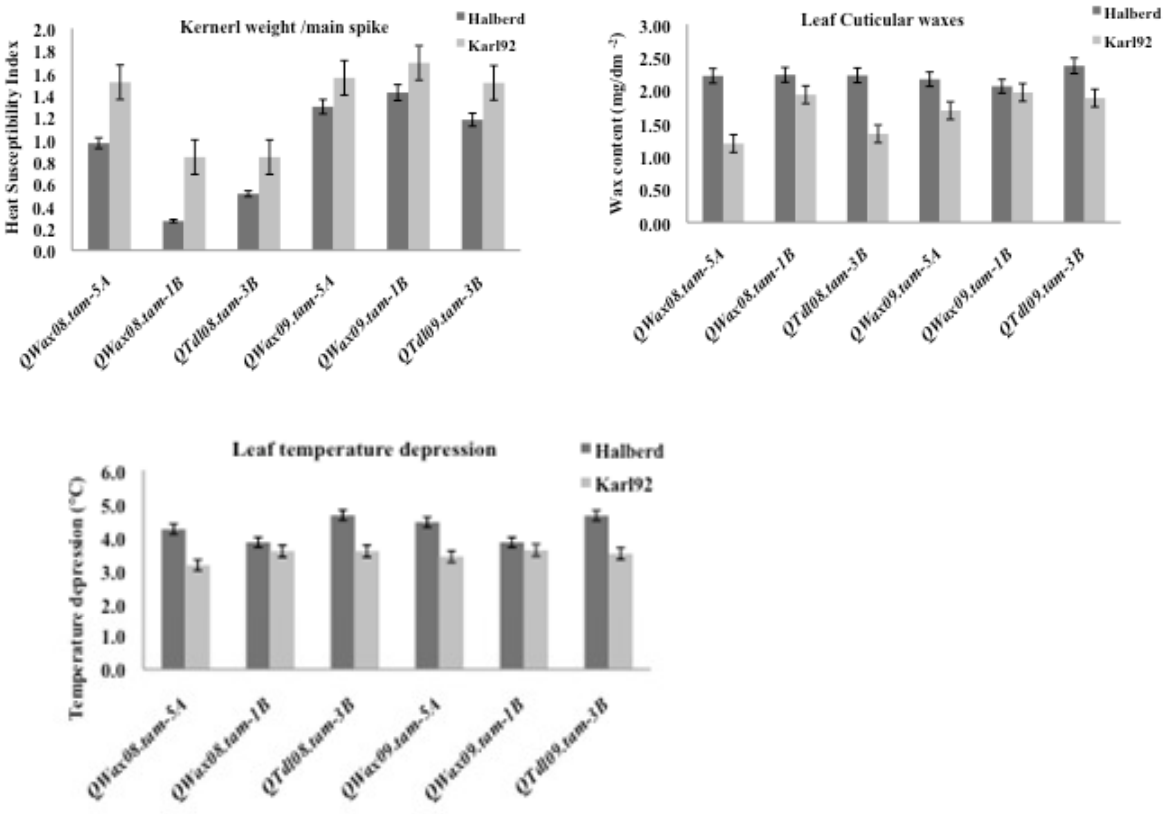


Fig. 10 Mean allele effect values for the QTL *QWax.tam-5A*, *QWax.tam-1B* and *QTdl.tam-3b* for HSI kernel weight per main spike, leaf cuticular wax and leaf temperature depression

3.4.5. *Stable QTL detected in 2008 and 2009*

Stable QTL for HSI of kernel weight, Td of leaf and spike, leaf cuticular waxes are summarized in Table 15. A stable QTL for HSI of kernel weight was detected during both short-term and long-term heat stress conditions on the chromosome 1B. This QTL region had previously reported to be linked to HSI of kernel weight (Mason et al. 2010), yield (Yang et al. 2002b) and grain filling duration (Kuchel et al. 2007b) during high temperature stress. The QTL on 1B is interesting as it is consistent and also co-localizes with other physiological and yield components (Fig. 11). A QTL for flag leaf length was also detected on 1B that was consistent in 2008 and 2009.

QTL for leaf temperature depression was detected on 3B that was consistent in both 2008 and 2009. The QTL had similar additive effects and explained 9% of the phenotypic variation. This QTL also co-localized with HSI for single kernel weight and spike temperature depression (Fig. 11).

The region on the long arm of chromosome 5A is also a very interesting region. Stable QTL for leaf and spike Td were detected in both years. A pleiotropic association between the HSI and temperature depression is present in this region (Fig. 11). In a previous of heat tolerance study in a 'Halberd' x Cutter RIL population also detected QTL for HSI of kernel weight and single kernel weight on 5AL. This region is known to contain the vernalizing gene *Vrn1A*. The pleiotropic associations between HSI and temperature depression and possible interaction with the *Vrn1A* at this locus require further study.

Table 15 Summary of QTL detected in the 'Halberd' x 'Karl92' population for HSI of main spike yield components, leaf cuticular waxes, leaf/spike temperature depression, flag leaf length, and flag leaf width that were consistent in 2008 and 2009

Year	Chromosome	QTL	Marker	LOD	R^2	Additive[b]	Positive allele
2008	1B	*QHkwm.tam-1B*	gwm153	3.93	0.101	-1.19	Halberd
2009	1B	*QHkwm.tam-1B*	wmc419	3.11	0.096	-0.34	Halberd
2008	1B	*QFll.tam-1B*	wmc156	3.08	0.038	1.31	Halberd
2009	1B	*QFll.tam-1B*	gwm268	3.32	0.078	1.07	Halberd
2008	3B	*QTdl.tam-3Bc*	barc84	2.75	0.093	0.44	Halberd
2009	3B	*QTdl.tam-3B*	wmc164	3.03	0.085	0.37	Halberd
2008	5A	*QWax.tam-5A*	gwm205	3.81	0.121	0.016	Halberd
2009	5A	*QWax.tam-5A*	wmc713	3.11	0.096	0.0165	Halberd
2008	5A	*QTdl.tam-5A.1*	gwm154	3.17	0.113	-0.60	Karl92
2009	5A	*QTdl.tam-5A.1*	barc186	3.9	0.165	-0.58	Karl92
2008	5A	*QTds.tam-5A.1*	gwm126	6.74	0.146	0.68	Halberd
2009	5A	*QTds.tam-5A.2*	gwm595	4.61	0.088	-0.56	Karl92

[a] LOD thresholds were estimated in QTL Cartographer v2.0 using 1000 permutation

[b] Additive effect of allele substitution

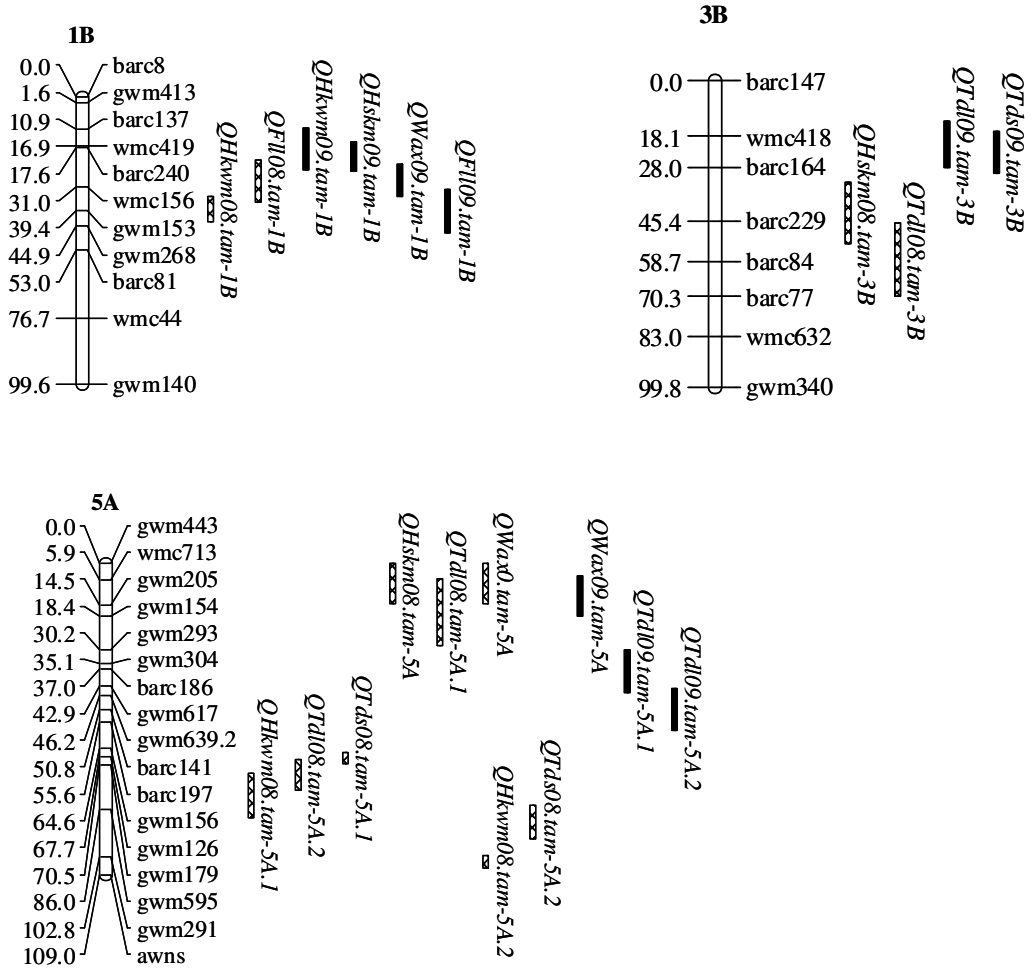


Fig. 11 Co-localization of QTL in the three chromosomes 1B, 3B and 5A for the HSI of yield components, leaf cuticular waxes and leaf and spike temperature depression

3.5. Conclusions

Leaf cuticular waxes were associated with leaf temperature depression and HSI of yield components during short-term heat stress conditions, but failed to show any major associations during long-term heat stress. Of the three QTL detected for flag leaf wax content one QTL was consistent with a previously identified non-glaucousness loci and the other two were pleiotropic with the HSI and leaf temperature depression. Cooler leaf and spike temperatures were associated with improved heat tolerance under both treatment regimes. QTL for temperature depression and HSI co-localized at several loci. Combining genetic loci associated with HSI, Td and leaf cuticular waxes may improve tolerance to high temperature stress.

CHAPTER IV

QTL MAPPING OF LEAF CUTICULAR WAX CONTENT, TEMPERATURE DEPRESSION AND YIELD COMPONENTS UNDER FIELD CONDITIONS

4.1. Introduction

Wheat is the most widespread cereal occupying an estimated 200 million hectares of land globally (Hodson and White 2009). Though wheat is primarily a temperate crop, it is also grown in the tropical and subtropical regions in the world. One of the major limitations of wheat growing in the tropical and sub-tropical region is high temperature stress. Wheat is sensitive to temperature increases. It has been reported that a 1°C temperature increase during grain filling shortens this period by 5% and reduces grain yield proportionally (Lawlor and Mitchell 2000). Although traditional breeding efforts have resulted in release of the germplasm adapted to high temperature environments, (Reynolds et al. 2001) the utilization of physiological and genetic approaches may enhance the understanding of high temperature stress tolerance and increase the pace of future gain. The genetic variability in the heat tolerant lines may result from physiological adaptive and stress tolerance mechanisms. The various physiological mechanisms that may contribute to heat tolerance are higher photosynthetic rates, stay green, membrane thermostability, canopy temperature depression and stomatal conductance (Reynolds et al. 2001). The study conducted in Chapter III associated cooler canopies with leaf cuticular waxes and improved heat tolerance under controlled

greenhouse environments. Physiological traits such as canopy temperature depression are being recognized as a potential tool for selection in breeding for heat tolerance.

Since heat tolerance is quantitative in nature, a genetic dissection of the traits associated with heat tolerance is essential to improve our understanding of the genetic basis of heat tolerance. Potential loci controlling the quantitative traits associated with heat tolerance have been reported in a few studies. QTL mapping studies identified loci on 1B and 5A that explained 23% of the variation in grain-filling duration under long-term high temperature stress (Yang et al. 2002a). The locus on 1B was also associated with yield under high temperature stress in a separate study (Kuchel et al. 2007b). A relatively few studies have examined the QTL associated with physiological traits. Mason et al. (2010) reported QTL associated with heat susceptibility index and canopy temperature depression on chromosome 1B, 5A and 6D. Genetic loci associated with heat tolerance, canopy temperature depression and leaf cuticular waxes were defined in the previous chapter of this dissertation. The study in Chapter III was carried out under controlled environmental conditions.

The specific objectives of this study were, 1) to characterize heat tolerance in the Halberd x Karl92 population in the field conditions 2) to determine the association of the canopy temperature depression and leaf cuticular waxes with yield and yield components under natural stress conditions and 3) identify stable loci associated with yield and physiological traits that will contribute to improved heat tolerance under field conditions.

4.2. Materials and methods

4.2.1. *Plant material*

A population of 121 recombinant inbred lines (RIL) was derived from a cross between heat tolerant spring wheat cultivar 'Halberd' and heat susceptible winter wheat cultivar 'Karl 92'. The initial crosses were developed in the greenhouse in 2003 in College Station, Texas. The lines were advanced by single seed descent to F5 and bulked to create 121 F_2F_6 (Mason et al. 2010). The F7 and the F8 generations were evaluated in the field in 2009 and 2010.

4.2.2. *Field trials*

A subset of the RIL lines (n=118) and the parent cultivars ‘Halberd’ and ‘Karl92’ were planted in the field in 2009 and 2010 under non-irrigated conditions. In 2009 the lines were sown in College Station (TX). In 2010, the lines were planted in two locations College Station (TX) and Uvalde (TX). The lines were planted in a randomized complete block design with two replications per line. The plot size was 3.04 x 1.52m and seeded at 50g per plot. The fungicide TILT (Syngenta Crop Protection, Inc., Greensboro, NC) was applied to control leaf rust infections. Due to uneven stand in the late sowing conditions in 2009, the plots were not harvested.

4.2.3. *Leaf cuticular waxes*

A colorimetric technique was used for leaf wax extraction and estimation (Ebercon et al. 1977). At 10 DAP four flag leaves were collected from each plot. Four leaf discs of 1cm diameter were punched from each flag leaf and placed in a glass vial. The leaf wax was extracted with HPLC grade chloroform for 30 sec and vacuum dried. The extracted wax

was oxidized with 300µl acidic potassium dichromate ($K_2Cr_2O_7$) in a water bath for 30 minutes. After cooling, 700µl of de-ionized water was added to each sample and allowed to develop color for an hour. The optical density of each sample was recorded at 590nm. A standard curve from randomly collected wheat flag leaf samples was used to estimate the amount of leaf wax amounts. The flag leaf wax content was calculated against the leaf area. It was expressed in mg/dm^2 units (Ebercon et al. 1977).

4.2.4. *Temperature depression*

Canopy temperatures were taken with a handheld infrared thermometer (Model AG-42, Teletemperature Corp, Fullerton, CA). The temperature depression (TD) was estimated as the difference between the air temperature and the canopy temperatures. Leaf and spike temperatures were recorded multiple times during grain filling and the TD was averaged for a single measurement per plot.

4.2.5. *Yield component and agronomic trait measurement*

Fifty spikes were randomly collected from each plot for estimation of the yield components. The whole plot was harvested to estimate yield and test weight. The yield components estimated were spike density (Spm), kernel number per spike (Kns), kernel weight per spike (Kws), kernel number per meter square (Knm), kernel weight per meter square (Knm). Days-to-flowering was estimated as the difference between the date of planting until the emergence of 50% of inflorescence in each plot. Grain-filling duration was measured as the number of days between the flowering date until the date of senescence of 90% of the inflorescence. Days-to-maturity was estimated as the time of

planting to 90% of senescence of inflorescence in each plot. Plant height was measured from the soil surface to the top of each plot. Flag leaf length and width were also measured in each plot. The symbols for phenotypic traits and the environments in the study are summarized in Table 16.

Table 16 Symbols for phenotypic traits and environments

Trait	**Symbol**	**Method of Measurement**
Days to flowering (days)	Hdg	Period of time from planting to emergence of 50% of inflorescences in each plot
Days to maturity (days)	Mat	Period of time from planting to 90% senescence in each plot
Grain-filling duration (days)	Gfd	Period of time from heading to 90% senescence in each plot
Kernel number per spike	Kns	Estimated from 50 individual spikes from each plot
Kernel weight per spike (g)	Kws	Estimated from 50 individual spikes from each plot
Single kernel weight (mg)	Skw	Estimated from kernel weight per spike/kernel number per spike
Spike density (spike/m^2)	Spm	Estimated 50 individual spikes from each plot
Kernel number per meter square	Knsm	Estimated from kernel number per spike and spike density
Kernel weight per meter square (g)	Kwsm	Estimated from kernel weight per spike and spike density
Test weight (g)	Twgt	Measure on a volume basis
Yield (kg/ha)	Yld	Total plot yield
Leaf /spike temperature depression (°C)	Tdl/Tds	Difference between air temperature and leaf/spike temperature
Leaf cuticular wax	Wax	Estimated by Colorimetric technique (Ebercon et al. 1977)

Environment	**Year**	**Location**
CS2009	2009	College Station, Texas
CS2010	2010	College Station, Texas
UVL2010	2010	Uvalde, Texas

4.2.6. *Molecular mapping and QTL analysis*

A genetic linkage map for the Karl92 x Halberd population was constructed with 189 markers and one phenotypic marker (Mason et al. 2010). The parent cultivars 'Halberd' and 'Karl92' were screened with 623 wheat SSR markers. Data from each environment was used for QTL mapping. In addition least square means (LSMEANS) were estimated from the combined data analysis and used for QTL mapping.

Quantitative trait loci (QTL) analyses were done in QTL Cartographer version 2.5 (WINQTL, Wang et al. 2007). A single marker analysis was initially used to identify the genetic markers significantly associated with the phenotype traits. Composite interval mapping (CIM) was then used to determine the QTL positions and effects. A 1000 permutation test was used to determine the LOD threshold for each trait at a significance level of P=0.05. A forward and backward regression method (p=0.05) with a10cm Window was used to identify QTL in CIM. A QTL was declared significant when it was detected in at least one environment as well as with the combined LSMEANS data. The QTL were designated based on the nomenclature in the catalog for gene symbols for wheat (http://wheat.pw.usda.gov/ggpages/wgc/98/), consisting of a "Q" followed by the trait name, institution designation, and chromosome assignment. Mapchart 2.2 software was used for generating genetic linkage maps and graphical presentation of QTL (Voorrips 2002).

4.2.7. *Statistical analysis*

Each year and location was treated as a separate environment. A combined analysis of variance was estimated using mixed model analysis), considering genotypes, environment and replications as random variable. The least square means (LSMEANS) for each trait was estimated. Variance components were used to estimate broad sense heritability by the following equation: $\sigma^2_G / (\sigma^2_G + \sigma^2_{GE/e} + \sigma^2_{E/re})$, where σ^2_G, σ^2_{GE}, σ^2_E are the genotypic variance, genotype x environmental variance and error variance respectively. Pearson's correlation was used for correlation analysis of all traits. Statistical software SAS Version 8.2 was used for all procedures (SAS Inst. Inc. 1990).

4.3. Results

The two locations College Station and Uvalde had subtropical environments with mild winters and hot summers. The average temperatures from flowering until grain-filling duration (mid-April – May) were 28°C to 32°C in College Station (in 2008 and 2009) with temperatures reaching up to 35°C on some days (www.weatherunderground.com). In Uvalde the average temperatures were 30.5°C with maximum temperatures of 32°C during flowering and grain filling (www.weatherundeground.com). The plots in College Station were treated with fungicides to control leaf rust infection. In Uvalde fungicide was applied to control both yellow rust and leaf rust infection.

4.3.1. *Phenotypic data in the three locations*

The yield and yield components were estimated for the parent cultivars and the RIL population in the three environments and presented in Table 17. 'Halberd' flowered earlier than 'Karl92' but had a longer grain filling duration. 'Halberd' had a higher kernel weight per spike, single kernel weight and spike density in all the three environments. The average yield of 'Karl92' was higher in CS2009 and may have resulted from the higher kernel number per spike in 2009. On average the yield and yield components for the parent cultivars and the RIL population was higher in 2009. The RIL population had significant genotypic variation for all the yield traits (Table 18). Significant environmental and GxE variation were also observed for all the yield traits estimated in the RIL population. Broad sense heritability estimates ranged from high to low in the yield traits (Table 18). High heritability was estimated for days to maturity ($H^2 = 0.89$), days to flowering ($H^2 = 0.74$) and single kernel weight ($H^2 = 0.72$). The heritability estimates were intermediate ($H^2 = 0.30$ to 0.60) for grain filling duration, yield, test weight and spike density. The yield traits kernel number per spike ($H^2 = 0.25$) and kernel weight per spike ($H^2 = 0.07$) had low heritability. LSMEANS were estimated for all the traits in the parent cultivars and RIL population (Table 19). The LSMEANS for all the traits in the RIL population were normally distributed except for days to flowering (Fig. 12). Transgressive segregation was observed in all traits.

Table 17 Mean and range of trait values for parental cultivars and Halberd x Karl 92 RIL measured in College Station, Texas and Uvalde, Texas in 2009 and 2010

Trait	CS 2009						CS 2010						UVL 2010					
	Halberd	**Karl 92**	**RIL**	**sd**	**Min**	**Max**	**Halberd**	**Karl 92**	**RIL**	**sd**	**Min**	**Max**	**Halberd**	**Karl 92**	**RIL**	**sd**	**Min**	**Max**
Days to heading	126.0	142.0	125.1	12.5	114.0	155.0	124	129	123.8	4.7	118	144	133	137	132.5	4.2	125	140
Grain filling Duration	42.0	31.0	42.9	9.2	25.0	73.0	38	32	33.5	4.3	25	37	-	-	-	-	-	-
Days to maturity	168.0	173.0	167.9	7.5	157.0	187.0	162	161	164.5	5.4	149	167	-	-	-	-	-	-
Yield (kg/ha)	2979.0	3043.3	2637.9	749.9	878.4	4503	2263	2173	2049	519	756	3535	2492	2273	2296	607	703	4567
Spike density (m^{-2})	271.8	250.6	252.5	75.1	82.1	498.2	241	220	240	78	105	492.2	298.6	243.5	301.7	77.3	106.7	541.3
Kernel weight per spike (g)	1.10	1.22	0.99	0.2	0.48	1.5	0.92	0.91	0.92	0.21	0.36	1.47	0.88	0.77	0.69	0.13	0.35	1.08
Kernel number per spike	33.2	48.0	33.3	6.4	19.5	47.6	34.98	31.63	33.06	6.01	15.5	49.72	31.88	28.87	28.41	4.15	17.12	39.9
Single kernel weight (mg)	33.0	27.2	30.4	2.9	24.8	39.5	29.5	28.7	29.1	2.3	20.8	37.8	27.6	26.5	26.4	0.03	23.4	35.1
Test weight	55.1	55.0	54.5	1.6	47.5	57.5	55.9	55.8	53.56	5.32	48.60	58.1	56.6	56.3	55.4	2.2	49.9	59.1
Wax (mg/dm^2)	2.43	1.94	2.43	0.04	1.42	3.79	2.64	2.04	2.68	0.07	1.53	3.49	2.47	1.79	2.28	0.8	1.51	3.42
Temperature depression leaf (°C)	4.45	2.55	2.37	1.3	-1.4	5.1	3.84	2.74	2.83	1.1	0.04	4.14	3.0	1.9	2.35	1.1	0.256	3.0
Temperature depression spike (°C)	-	-	-	-	-	-	3.03	2.50	2.12	1.01	-0.21	4.64	1.3	0.9	1.81	0.9	-0.46	2.08

CS2009-College Station, TX 2008-2009, CS2010- College Station, TX 2009-2010 and UVL2010-Uvalde, TX 2009-2010

Table 18 Variance component and broad sense heritability estimates from the combined analysis of all environments in the RIL population

	Variance component[a]					
Trait	**H^2**	**G**	**E**	**GxE**	**Rep(E)[d]**	**Residual**
Heading (days)	0.74	0.38**	0.16**	0.34**	0.003	0.11
Grain-filling duration (day)	0.60	0.19**	0.35**	0.36*	0.004*	0.07
Maturity (days)	0.89	0.14**	0.78**	0.03**	0.004**	0.036
Yield (kg/ha)	0.65	0.311**	0.33**	0.14**	0.17**	0.035
Spike density (spike/m^2	0.42	0.12**	0.08**	0.25**	0.05**	0.47
Kernel weight per spike (g)	0.07	0.02**	0.37**	0.33**	0.021**	0.25
Kernel number per spike	0.25	0.07*	0.17**	0.35**	0.01*	0.40
Single kernel weight (mg)	0.72	0.24**	0.36**	0.17**	0.02*	0.19
Test Weight	0.46	0.14**	0.13*	0.30**	0.01*	0.01
Wax	0.13	0.02*	0.14**	0.31*	0.07*	0.16
TDL	-	0.06*	0.20*	0.00	0.09*	0.64
TDH	-	0.07*	0.32*	0.00	0.20*	0.40

$H^2 = \sigma_g^2 / \sigma_g^2 + \sigma^2_{gxe/e} + \sigma^2_{error/re}$

[a] Variance component of each effect divided by the total of all variance components G, genotype, E, environment, GxE, genotype x environment

[d] replications nested within environment

*Significant a P=.05, **Significant at P=.01, ***Significant at P=.001

Table 19 Mean, range and heritability estimates for each trait in the parent cultivars and RIL population across environments based on the least square mean (LSMEAN) values

	Parents		RIL Population			
Trait	**Halberd**	**Karl92**	**Mean**	**Min**	**Max**	**H^2**
Days to flowering	127	135	126.2	119	141	0.74
Grain filling Duration	34.5	27.5	34.3	25.5	48.2	0.60
Days to maturity	159.5	162	158.7	148	170.25	0.89
Yield (kg/ha)	2596	2188	2260	873	3477.2	0.65
Spike density (m^{-2})	244.6	228.92	274.8	159	448	0.42
Kernel weight per spike (g)	0.951	0.915	0.901	0.856	1.13	0.07
Kernel number per spike	32.99	35.83	31.6	25.83	39.87	0.25
Single kernel weight (mg)	28.8	26.5	28.7	25.6	35	0.72
Test Weight	56.48	53.35	54.48	50.85	57.9	0.46
Wax (mg/dm^2)	2.53	2.04	2.75	1.437	3.08	0.13
Temperature depression leaf (°C)	3.03	2.24	2.69	1.01	4.3	-
Temperature depression spike (°C)	1.62	1.27	1.65	0.58	3.1	-

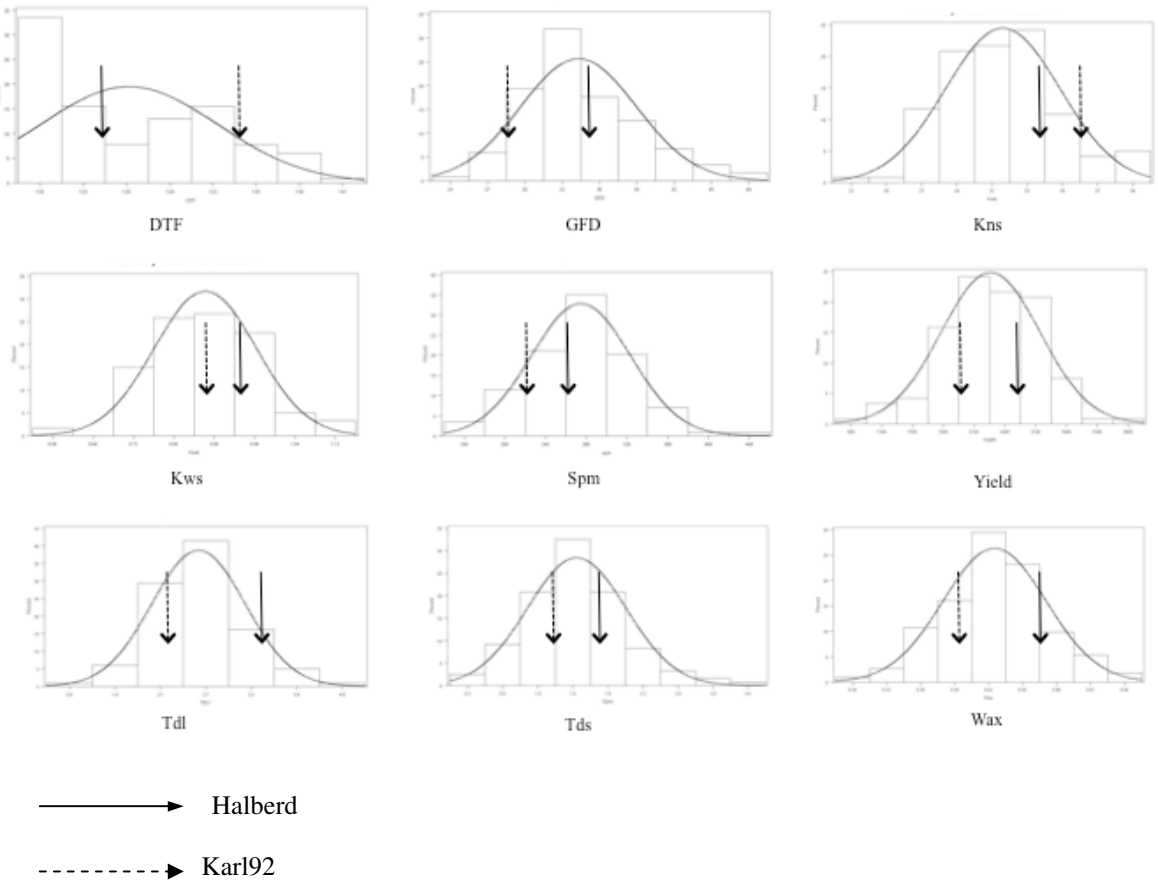


Fig. 12 Normality curves derived from the least square mean (LSMEANS) values estimated across the environments for the following traits: days to flowering (DTF), grain filling duration (GFD), kernel number per spike (Kns), kernel weight per spike (Kws), spike density (Spm), yield, temperature depression leaf (Tdl), temperature depression spike (Tds), and leaf cuticular wax content (Wax)

4.3.2. *Physiological traits*

The physiological traits evaluated in field conditions were leaf temperature depression, spike temperature depression and leaf cuticular waxes. The parent cultivars 'Halberd' and 'Karl92' had significant variation for the all the physiological traits in the study. 'Halberd' had consistent higher flag leaf cuticular wax content in all the environments (Table 17). Leaf cuticular wax content in the RIL population ranged from 1.4 mg / dm^{-2} to 3.1 mg / dm^{-2} with some lines showing higher leaf cuticular wax content than 'Halberd' (Fig. 12). A combined analysis shows significant genotypic, environmental and G x E variation in leaf cuticular waxes (Table 18). Environment had a strong effect on leaf cuticular wax content in the RIL population. The broad sense heritability of the leaf cuticular waxes was 0.13 (Table 18).

Leaf /spike temperatures were recorded three times during grain filling and the mean leaf /spike temperature depression was estimated for each environment (Table 17). 'Halberd' had cooler canopies than 'Karl92' in all the three environments. The leaf and spike temperature depression in 'Halberd' was 3.03°C and 1.62°C respectively while in 'Karl92' it was 2.24°C and 1.24°C respectively. The RIL population had significant genotypic variation for leaf and spike temperature depression (Table 18). The leaf and spike temperature depression was normally distributed. The leaf temperature depression in the RIL population ranged from 1°C to 4°C (Table 17). The spike temperature depression ranged from 0.31°C to 3.0°C (Table 17). Significant environmental and G x E effect was observed for leaf temperature depression. No significant G x E was observed for spike temperature depression.

The temperature depression in UVL2010 was lower than CS2009 and CS2010 as the mean air temperature was 30-32°C in Uvalde in contrast to the 33-35°C in College Station.

4.3.3. *Correlation analysis*

Pearson's correlation analysis among the yield traits and the physiological traits are presented in Table 20. Yield was highly correlated with spike density, kernel weight per spike, kernel number per spike, single kernel weight and grain filling duration. Days to flowering were negatively correlated with all yield components. The recombinant inbred lines that flowered later were exposed to high temperature stress (>30°C), at early reproductive stages, which may have resulted in further yield reductions. Grain filling duration was positively correlated with all yield components. A significant negative correlation was observed between spike density and kernel weight and kernel number per spike. Thus implying that increased tiller number reduced kernel number and kernel weight.

Leaf temperature depression was positively correlated with leaf cuticular waxes. Thus increase in leaf cuticular wax content contributed to cooler leaf temperatures. Leaf temperature depression showed a low correlation with kernel weight per spike and kernel number per spike. Spike temperature depression was positively correlated with kernel number per spike, kernel weight per spike and single kernel weight spike. A positive correlation was observed between grain filling duration and spike temperature depression indicating that reduction in spike temperature resulted in increased grain filling duration. Leaf cuticular wax content had a low correlation of 0.126 ($p=0.05$) with

kernel number per spike. Leaf cuticular wax content did not correlate with any other yield component.

Table 20 Pearson's correlation coefficients for yield and physiological traits

	Tdl	**Tds**	**Wax**	**Yield**	**Spm**	**Krws**	**Krns**	**Skrw**	**Dtf**	**Gfd**
Tdl	-	0.504**	0.146*	0.091	-0.043	0.114*	0.098*	0.07	0.122**	-0.158**
Tds		-	0.032	0.068	0.017	0.158*	0.126*	0.137*	-0.122**	0.158*
Wax			-	0.053	-0.047	0.049	0.117*	0.066	-0.09*	-0.07
Yield				-	0.739**	0.219**	0.171**	0.449**	-0.156**	0.522**
Spm					-	-0.299**	-0.419**	0.015	-0.092*	0.478**
Kws						-	0.829**	0.676**	0.061	-0.023
Kns							-	0.165*	-0.141**	0.176**
Skrw								-	-0.348**	0.341**
Dtf									-	-0.411**
Gfd										-

*Significant at p=0.05, ** Significant at p=0.01
Tdl-Temperature depression leaf, Tds – Temperature depression spike, Wax – flag leaf cuticular wax
Spm- Spikes per meter square, Kws – kernel weight per spike, Kns – kernel
Skrw – Single kernel weight
Dtf – Days to flowering, Gfd – Grain filling duration

4.3.4. *Linkage mapping and QTL analysis*

The linkage map for Halberd x Karl92 was constructed with 190 SSR markers (further details in Mason et al. 2010). The map consisted of 21 linkage groups. Due to uneven distribution of markers some large gaps were present in the chromosomes 3D, 4A, 4D and 6D. The marker order though was conserved and in consensus with wheat genetic maps (Somers et al. 2004).

QTL analysis was performed with the trait data of individual environments and the LSMEANS estimate of each trait across the three environments. A QTL was declared significant when it was identified in the combined analysis and at least in one other individual environment. A single marker analysis was initially used to identify significant marker trait association. Composite interval mapping was then used to identify the significant QTL associated with the traits. Since single marker analysis does not determine the QTL position or effects, only the results from the CIM analysis will be presented and discussed.

QTL analysis identified 28 loci for yield and yield components (Table 21). A detailed summary of the QTL detected for each trait with its LOD, position and effects are summarized in Table 21. The high yielding parent 'Halberd' contributed favorable alleles to most of the loci. Six significant QTL were identified for yield, of which four QTL had favorable alleles from 'Halberd' and the other two from 'Karl92'. The two QTL for yield identified on 1D, *QYld.tam-1D.1* and *QYld.tam-1D.2* were set 20 CM apart and were identified in the combined analysis across the environments and the individual environments CS2009 and CS2010 respectively. 'Halberd' contributed

favorable alleles to both the QTL and each explained 6% and 9% of the total phenotypic variation. Another QTL on 5A though explained only 8% of the phenotypic variation but was identified in all three individual environments and across the environments. For the yield components two QTL were identified for kernel number per spike, four QTL for kernel weight per spike, four QTL for single kernel weight, three QTL for spike density, two QTL for kernel number per meter square, three QTL for kernel weight per meter square and four QTL for test weight. The two QTL identified for kernel number per spike *QKns.tam-2B* and *QKns.tam-4B* had favorable alleles from 'Karl92'. The QTL *QKns.tam-4B* had a high LOD score of 7.38 and explained 18% of the phenotypic variation. This QTL also co-localized with a QTL for kernel weight per spike *QKws.tam-4B*. The favorable allele for the QTL *QKws.tam-4B* was from 'Karl92' and explained 6% of the variation in kernel weight per spike. 'Halberd' contributed favorable alleles for two QTL for kernel weight per spike on chromosome 5A that co-localized with the QTL for yield and single kernel weight. The other two QTL for single kernel weight on 2D co-localized with the flowering time QTL identified in the same region. Three QTL were detected for spike density one each on 1A, 5B and 7B each explaining 9%, 13% and 15% of the phenotypic variation. 'Halberd' contributed favorable alleles to the QTL on 1A while 'Karl92' contributed to 5B and 7B. The two QTL detected for kernel number per meter square were on chromosome 3B and 6D and their positive allele was from 'Karl92'. The QTL on 3B explained 13% of the phenotypic variation and co-localized with the QTL for kernel weight per meter square and yield. There were two other QTL detected for kernel weight per meter square on

chromosome 5A that had favorable alleles from 'Halberd' and co-localized with QTL for kernel weight per spike, single kernel weight and yield.

Five QTL were detected for days to flowering, two of which *QDtf.tam-2D.1* and *QDtf.tam-2D.2* was located near to the *Ppd-D1* gene (photoperiod sensitivity). The QTL *QDtf.tam-2D.1* was identified in all three individual environments CS2009, CS2010 and UVL2010 and explained 14% of the phenotypic variation. The other QTL on chromosome 2D *QDtf.tam-2D.2* was identified in CS2009 and UVL2010 and explained 9% of the variation. The QTL *QDtf.tam-5B* was located in proximity of the vernalization gene *VrnB1* and was identified only in one CS2010. A QTL for grain filling duration on 2D co-localized with the flowering time QTL *QDtf.tam-2D.1* (Fig. 13). QTL for grain filling duration were also detected on chromosome 5A. Though the two QTL for grain filling were contributed by different parents and were present only in one other individual environment they co-localize with the yield and yield component loci identified on the same chromosome (Fig. 13). Two QTL were detected for days to maturity. One QTL on 2D co-localized with the flowering time loci *QDtf.tam-2D.1* loci. The second QTL on 4B had a favorable allele from 'Karl92' and explained 9% of the phenotypic variation.

Table 21 QTL detected in the Halberd x Karl 92 mapping population (n=121) for yield, yield components and agronomic traits based on the LSMEAN values estimated across the environments

QTL (LOD threshold[a])	Marker	LOD	R^2	Additive[b]	Positive allele	Environments detected[c]
Days to Flowering (3.00)						
QDtf.tam-2D.1	gwm484	6.43	0.142	2.4	Halberd	CS2009, CS2010, UVL2010
QDtf.tam-2D.2	wmc601	3.58	0.091	2.9	Halberd	CS2009, UVL2010
QDtf.tam-4A	barc78	4.16	0.112	2.29	Halberd	UVL2010
QDtf.tam-5B	wmc73	3.93	0.071	-1.67	Karl92	CS2010
QDtf.tam-7A	wmc603	4.83	0.124	2.23	Halberd	CS2009
Grain filling duration (4.12)						
QGffd.tam-2D	gwm484	3.10	0.08	1.43	Halberd	CS2009, CS2010
QGfd.tam-5A	barc186	2.57	0.069	1.34	Halberd	CS2010
QGfd.tam-5B	barc142	3.71	0.208	-2.19	Karl 92	CS2010
Days to Maturity (3.54)						
QDtm.tam-2D	gwm484	7.49	0.316	3.43	Halberd	CS2009, CS2010
QDtm.tam-4A	wmc707	3.03	0.095	1.99	Halberd	CS2010
Kernel number / spike (4.10))						
QKns.tam-2B	barc91	4.69	0.085	1.02	Karl 92	CS2009
QKns.tam-4B	wmc89.1	7.38	0.183	-1.43	Karl 92	CS2010
Kernel weight /spike (4.26)						
QKws.tam-4B	gwm149	3.17	0.065	-0.027	Karl 92	UVL2010
QKws.tam-5A.1	gwm154	3.87	0.13	0.038	Halberd	CS2010
QKws.tam-5A.2	barc186	2.60	0.05	0.025	Halberd	CS2010, UVL2010
QKws.tam-6B	gwm508	2.60	0.058	-0.025	Karl 92	UVL2010
Single kernel weight (4.06)						
QSkw2.tam-2D.1	gwm484	4.2	0.089	-0.008	Karl92	CS2010
QSkw2.tam-2D.2	gwm382	4.22	0.095	0.009	Halberd	UVL2010
QSkw2.tam-5A.1	gwm154	5.56	0.149	0.0010	Halberd	CS2009, CS2010, UVL2010
QSkw2.tam-5A.2	barc186	5.65	0.123	0.0010	Halberd	CS2009 , UVL2010
Spike Density (2.54)						
QSpm.tam-1A	wmc93	3.76	0.094	17.57	Halberd	CS2010
QSpm.tam-5B	barc142	2.90	0.126	-17.87	Karl92	CS2010, UVL2010
QSpm.tam-7B	barc267	2.63	0.145	-19.54	Karl92	CS2009, CS2010
Kernel Number /m^{-2} (3.04)						
QKnm.tam-3B	barc229	3.29	0.127	-572.5	Karl92	CS2010.CS2009
QKnm.tam-6D	cfd76	3.59	0.071	-417.3	Karl92	UVL2010
Kernel weight /m^{-2} (3.34)						
QKwm.tam-3B	barc229	3.66	0.139	-18.01	Karl92	CS2009, UVL2010
QKwm.tam-5A.1	gwm154	3.83	0.115	17.12	Halberd	UVL2010
QKwm.tam-5A.2	barc186	3.51	0.063	13.45	Halberd	CS2009
Test weight (2.82)						
QTwgt.tam-2B	wmc257	3.39	0.079	0.4021	Halberd	CS2010
QTwgt.tam-2D	gwm484	7.48	0.174	-0.592	Karl 92	CS2009, CS2010

Table 21 (continued)

QTL (LOD threshold[a])	Marker	LOD	R^2	Additive[b]	Positive allele	Environments detected[c]
QTwgt.tam-4B	wmc89.1	4.2	0.082	0.411	Halberd	UVL2010
QTwgt.tam-5A	gwm126	3.15	0.075	-0.396	Karl 92	CS2009, UVL2010
Yield (3.48)						
QYld.tam-1D.1	gwm337	3.56	0.065	131.96	Halberd	CS2009
QYld.tam-1D.2	barc148	3.95	0.095	155.12	Halberd	CS2010
QYld.tam-3B	barc84	3.81	0.105	155.12	Halberd	CS2009, UVL2010
QYld.tam-5A.1	barc186	3.56	0.079	141.81	Halberd	CS2009. CS2010, UVL2010
QYld.tam-5A.2	gwm156	3.69	0.080	-172.3	Karl92	CS2010
QYld.tam-7D	barc76	3.66	0.0649	-130.4	Karl 92	CS2009

[a] LOD thresholds were estimated in QTL Cartographer v2.0 using 1000 permutation
[b] Additive effect of allele substitution
CS2009 –College Station 2009, CS2010-College Station 2010, UVL2010 –Uvalde 2010

4.3.5. *QTL mapping of physiological traits*

The QTL analysis for leaf temperature depression detected five QTL one each on 1A, 1B, 2D, 6A and 7D (Table 22). The QTL *QTdl.tam-2D* on chromosome 2D explained the largest proportion of phenotypic variation but was in close proximity to the *QDtf.tam-2D.2* or the flowering time loci. 'Halberd' contributed favorable alleles in the loci *QTdl.tam-1B*, *QTdl.tam-6A* and *QTdl.tam-1D* and QTL explained 7%, 10% and 9% of the total phenotypic variation. The QTL *QTdl.tam-1B* was detected in two individual environments CS2009 and CS2010. For spike temperature depression three QTL were detected on chromosomes 1B, 1D and 5A (Table 22). The QTL *QTds.tam-1D* on chromosome 1D was detected in two environments and explained 10% of the phenotypic variation. 'Halberd' contributed the favorable allele to *QTdl.tam-1D*.

Five significant QTL were detected for leaf cuticular waxes (Table 22). 'Karl92' contributed to the QTL *QWax.tam-1B* and it explained 13% of the variation.

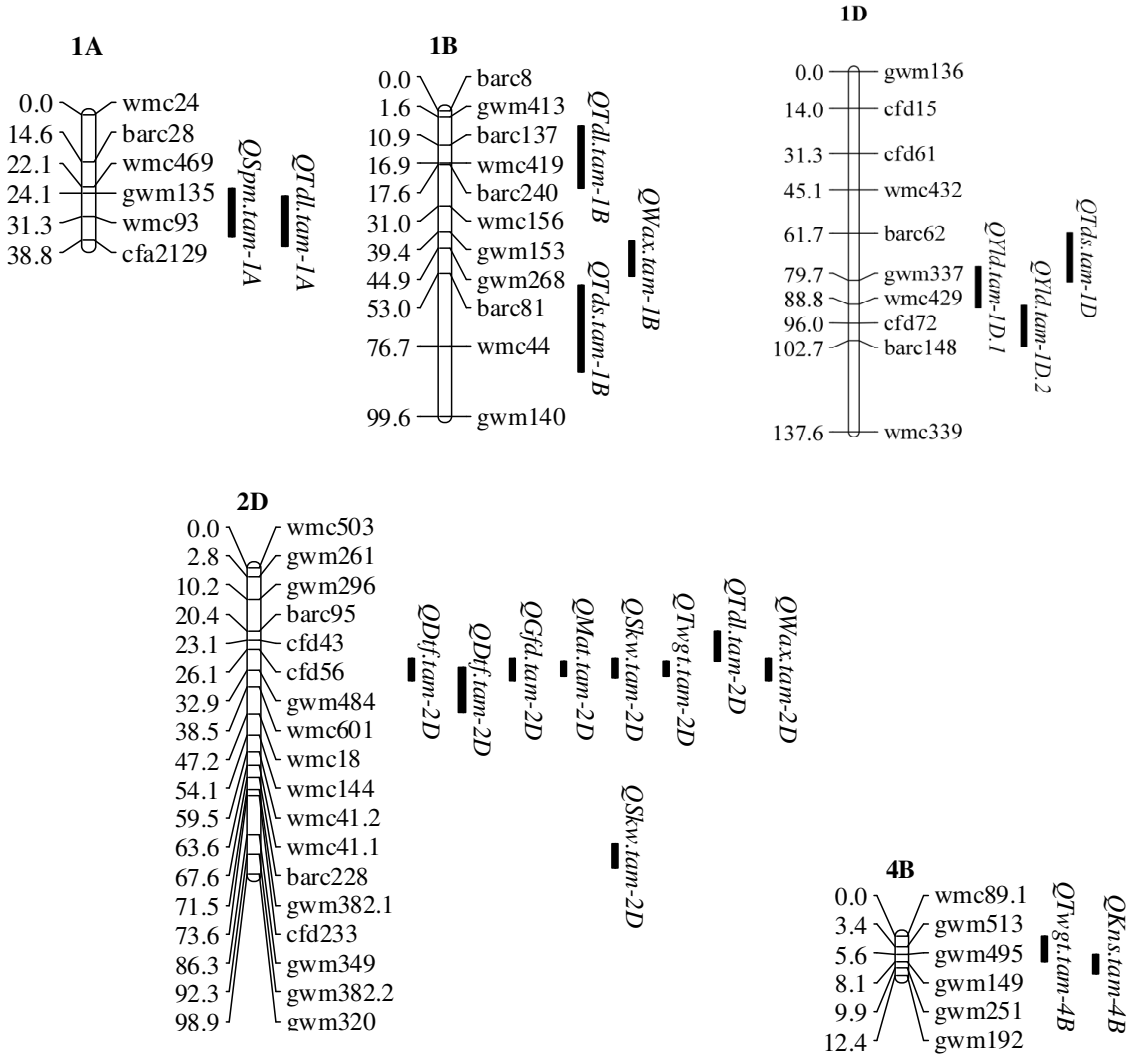


Fig. 13 Linkage map and QTL for the yield, yield components and physiological traits detected in the Halberd x Karl92 population. The QTL were annotated based on the trait name, linkage group and position on the chromosome and were presented as 2LOD intervals. The traits were abbreviated according to Table 16

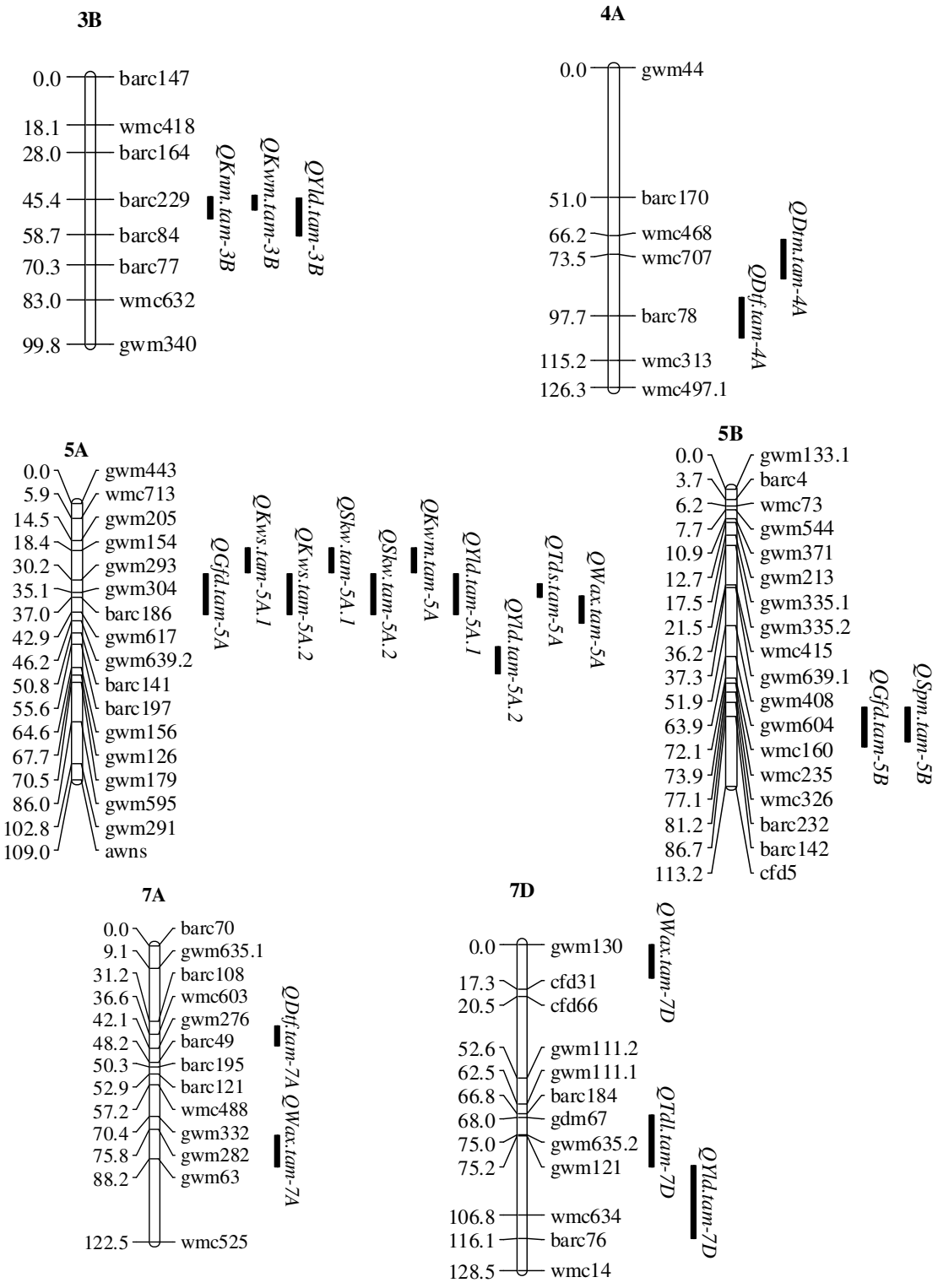


Fig. 13 (continued)

This QTL *QWax.tam-1B* has previously been associated with spike non-glaucousness (McIntosh et al. 2009) and also with leaf cuticular waxes in Chapter III of this dissertation. A significant QTL for leaf cuticular waxes was detected on chromosome 7A. 'Halberd' contributed the favorable allele to the QTL *QWax.tam-7A* and it explained 19% of the phenotypic variation. This QTL had a high LOD score of 6.64 but was identified only in one individual environment UVL2010. The QTL detected on chromosome 5A was detected in two of the individual environments and explained 9% of the phenotypic variation. The QTL on 7D *QWax.tam-7D* explained 9% of the variation and the favorable allele was from 'Halberd'.

Table 22 QTL detected in the Halberd x Karl 92 mapping population (n=121) for physiological traits based on the LSMEAN values estimated across all environments

QTL (LOD threshold[a])	Marker	LOD	R^2	Additive[b]	Positive allele	Environments detected[c]
Temperature depression leaf (4.05)						
QTdl.tam-1A	mwc469	4.54	0.058	-0.158	Karl92	CS2010
QTdl.tam-1B	barc240	3.68	0.0689	0.171	Halberd	CS2009, CS2010
QTdl.tam-2D	cfd56	6.48	0.191	-0.301	Karl92	CS2009, CS2010
QTdl.tam-6A	wmc417	4.23	0.100	0.212	Halberd	CS2010
QTdl.tam-7D	gdm67	4.31	0.096	0.200	Halberd	CS2010
Temperature depression spike (3.83)						
QTds.tam-1B	gwm140	3.72	0.129	-0.205	Karl92	CS2009
QTds.tam-1D	cfd62	3.97	0.0981	0.182	Halberd	CS2009, UVL2010
QTds.tam-5A	gwm304	3.96	0.0936	0.181	Halberd	CS2010
Leaf cuticular wax (4.13)						
QWax.tam-1B	gwm268	3.85	0.127	-0.169	Karl92	CS2009
QWax.tam-2D	gwm484	3.90	0.184	-0.020	Karl92	CS2009, CS2010
QWax.tam-5A	gwm639.2	3.26	0.094	-0.011	Karl92	CS2010, UVL2010
QWax.tam-7A	gwm63	6.64	0.193	0.021	Halberd	UVL2010
QWax.tam-7D	gwm130	3.41	0.089	0.014	Halberd	CS2010

[a] LOD thresholds were estimated in QTL Cartographer v2.0 using 1000 permutation, [b] Additive effect of allele CS2009 –College Station 2009, CS2010-College Station 2010, UVL2010 –Uvalde 2010

4.3.6. *Co-localization of QTL identified for yield and yield components and the physiological traits*

For most traits at least one QTL was detected on chromosome 2D that co-localized with the flowering time loci *QDtf.tam-2D /Ppd-D1* (Fig. 13). The gene *Ppd-D1* is a major photoperiod gene that has been linked to the marker gwm484 (Hanocq et al. 2004). The QTL for leaf cuticular wax content on 1B *QWax.tam-1B* co-localized with the leaf (*QTdl.tam-1B*) and spike temperature depression (*QTds.tam-1B*) QTL. The QTL *QTdl.tam-1B* was detected combined analysis and in CS2009 and CS2010 whiles the *QTds.tam-1B* and *QWax.tam-1B* were detected in combined analysis and in CS2009. The yield QTL *QYld.tam-1D.1* co-localized with the spike temperature depression *QTds.tam-1D*. A QTL for leaf temperature depression on long arm of chromosome 7D co-localized with yield QTL *QYld.tam-7D*. 'Halberd' contributed favorable allele to both QTL. Co-localization of several traits was detected on chromosome 5A. All yield traits (except for kernel number per spike and kernel number per meter square) and physiological traits had at least one QTL in the same region on chromosome 5A. Though the vernalization gene *VrnA1* is located on chromosome 5A this study does not detect flowering time loci on chromosome 5A. Two QTL for grain filling duration were identified on 5A that were also associated with the yield QTL and had their favorable allele from 'Halberd' implying a positive contribution of this region to yield under high temperature stress.

4.4. Discussion

4.4.1. *Phenotypic and physiological traits in field conditions*

The previous study in Chapter III of this dissertation was focused characterizing heat tolerance and mapping QTL associated physiological and phenotypic traits that contributed to short-term and long-term heat tolerance. The current study was conducted under field conditions and hence there was no control over the high temperature stress conditions. Although based on the temperature data available the RIL population was subjected to increasing temperatures (>30°C) during flowering and grain filling simulating a long-term heat stress conditions. The performance of the parent cultivars was similar to the controlled conditions experiments in the greenhouse. 'Halberd' had cooler canopies and yielded higher than 'Karl92' in the field. Similar results were also observed in previous studies were 'Karl92' was characterized as the heat susceptible line (Yang et al. 2002a; Mason et al. 2010) and 'Halberd' as the heat tolerant cultivar (Hays et al. 2007b, Mason et al. 2010). The flag leaf cuticular waxes at 10DAP were higher in 'Halberd' in all three environments. This was similar to the results found in Chapter II and Chapter III of this dissertation where the heat tolerant parent 'Halberd' had higher leaf cuticular wax content compared to the heat susceptible parent 'Karl92'.

There were significant genotypic variation for all yield and physiological traits in the RIL population. Although G x E variation was significant for all traits in the study, environmental variation appears to have a stronger effect. The broad sense heritability for the yield traits ranged from moderate to high except for kernel number per spike that had a low heritability of H^2= 0.07. Due to significant environmental and G x E variation

the heritability of flag leaf cuticular waxes was low (H^2= 0.13). The yield, plant developmental and physio-morphological traits were normally distributed in the RIL population except for days to flowering. Transgressive segregation was observed in most of the traits.

There was a negative correlation between the days to flowering and all yield traits except kernel number per spike. The parent cultivars had a week difference in their flowering time with 'Halberd' flowering in 127days and 'Karl92' flowering in 135 days. The RIL population was segregating for flowering time (Fig.12) and had a difference of 30 days between the early and late flowering lines (Table 17). The late flowering lines were exposed to high temperature stress at early reproductive stage that lead to further reduction in yield. The effect of flowering time was also evident in the QTL analysis where at least one QTL for most traits under study co-localized with the flowering time QTL. The results are in agreement with the previous studies that have established the importance of early flowering as a heat avoidance mechanism (Araus et al. 2008). In the previous study in Chapter III under controlled conditions in the greenhouse all the lines were treated at the same reproductive stage of 10 DAP and thus the effects of flowering time on the QTL analysis was not significant.

The leaf cuticular waxes correlated significantly with leaf temperature depression (r=0.15) but failed to show any correlations with any yield or yield components. Leaf temperature depression correlated with the kernel number and kernel weight per spike but not with yield itself. The association of leaf temperature depression with kernel number has also been previously shown in Chapter III of this thesis. The association of

temperature depression with flowering time may have masked the effects of cooler canopies on yield under stress conditions.

4.4.2. *QTL analysis and co-localization with previous studies*

The most stable QTL detected in the study was for flowering time on chromosome 2D. The QTL was in proximity with the photoperiod gene *Ppd-D1* and was present in the combined analysis and in all three-field environments. This QTL was also found in the greenhouse studies discussed in Chapter III. QTL for yield components, leaf temperature depression and leaf cuticular waxes were pleiotropically associated with the flowering time loci. Other studies have also reported pleiotropic association of QTL for yield and flowering time (Cuthbert et al. 2008; Mason et al. 2010). The effects of segregating flowering time may also have been a reason for the lack of detection other stable QTL.

Two QTL for grain yield were located on chromosome 1D at a distance of less than 20cM and explained an overall of 16% of the phenotypic variation (Table 21). The QTL also co-localized with the spike temperature depression QTL *QTds.tam-1D*. The QTL *QTds.tam-1D* was detected in CS2010 and UVL2010 and across the environments and explained 9% of the phenotypic variation. A mean allele contrast analysis for the yield QTL and spike temperature depression QTL located in this region is presented in Fig. 14. The 'Halberd' allele at this locus was associated with higher yield and spike temperature depression in all the environments.

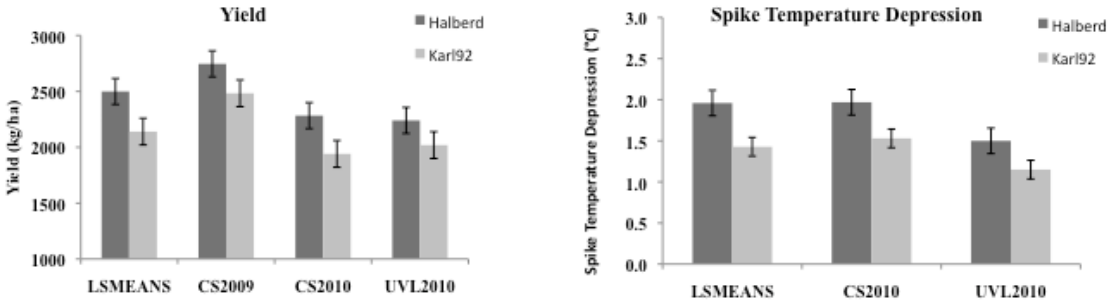


Fig. 14 Mean allele values for yield and spike temperature depression in the RIL having either 'Halberd' or 'Karl92' allele for the marker barc62 that was closely associated with the QTL *QTds.tam-1D*

Stable QTL for leaf cuticular wax content were detected in the field studies and the greenhouse studies. The QTL *QWax.tam-1B* was detected in the combined field analysis, CS2009 and in the greenhouse in 2009. The favorable allele for the QTL was from 'Karl92' in all the environments. A gene for spike non-glaucousness, *ws*, was mapped on chromosome 1BS in the cross *T. durum* cv. Langdon / *T. dicoccoides* acc. Hermon H52 and co-localized with *QWax.tam-1B* (McIntosh et al. 2009). Leaf and spike temperature depression QTL had a pleiotropic association at the *QWax.tam-1B* loci. A previous study mapped leaf non-glaucousness loci *Iw3672* to the distal end of chromosome 2DS (Liu et al. 2007). Association of the QTL *QWax.tam-2D* and the non-glaucousness loci *Iw3672* was not possible due to its co-localization with flowering time QTL *QDtf.tam-2D* and absence of markers linked to *Iw3672* in the Halberd x Karl92 map. The QTL for leaf cuticular waxes detected on chromosome 7D was located at a

close proximity with adult plant rust resistance gene Lr34. The Lr34 protein was reported to be similar to adenosine triphosphate binding cassette (ABC) transporter protein (Krattinger et al. 2009). ABC transporters have been suggested to be involved in transportation of leaf cuticular waxes (Bird et al. 2007; Samuels et al. 2008). Although no association of leaf cuticular waxes and leaf rust in wheat has been reported yet but the role of leaf cuticular waxes in deterring pathogens is well known (Riederer 2006).

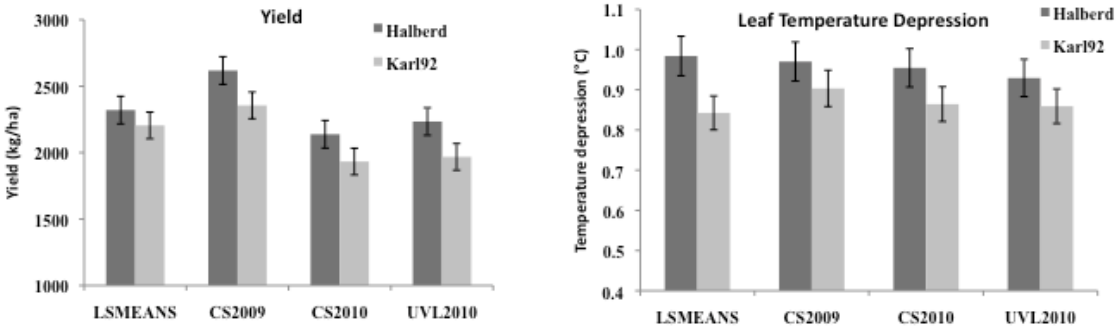


Fig. 15 Mean allele values for the traits associated with the marker barc186 on chromosome 5A

The chromosome 5A was associated with stable QTL for yield, kernel weight per spike, single kernel weight, kernel weight per meter square, test weight, leaf and spike temperature depression and leaf cuticular waxes. This region was also found to be

significantly associated with HSI of yield traits and leaf and spike temperature depression traits in the greenhouse studies discussed in Chapter III. The favorable parent allele in this region was similar for all the yield traits. Presence stable QTL for yield and physiological traits on chromosome 5A may suggest the association of this region to stress tolerance. A mean allele contrast analysis for the yield and leaf temperature depression that was significantly associated in this QTL region is presented in Fig. 15. Though significant differences were not observed in all environments, 'Halberd' allele increased yields and spike temperature depression at this locus.

On the long arm of chromosome 7D a QTL for leaf temperature depression and yield were co-localized. QTL associated with yield components and temperature depression has been previously reported in this region in a study with the same RIL population (Mason et al. 2010). Another stable QTL for yield was identified on chromosome 3B. This QTL co-localized with kernel number per meter square QTL *QKnm.tam-3B* and kernel weight per meter square QTL *QKwm.tam-3B*. This region on 3B was associated with QTL for single kernel weight and temperature depression in the greenhouse studies in Chapter III. Previous studies have also associated this region with yield and tiller number (Kumar et al. 2007) and yield, thousand kernel weight and spikes /m^{-2} (Mason et al. 2010). Pleiotropic associations of QTL for yield and physiological traits suggest the adaptive QTL.

4.5. Conclusion

Leaf cuticular wax content was associated with leaf temperature depression under the field conditions. Except kernel number per spike, leaf cuticular wax content had no

correlations with yield or yield components. . Leaf and spike temperature depression were associated with yield and yield components under field conditions and with HSI of yield components in the greenhouse study Flowering time had a major impact in the field study. QTL analysis detected QTL for leaf cuticular waxes, leaf temperature depression and yield components. Stable QTL for leaf cuticular waxes were detected on chromosome 1B and 5A. These QTL co-localized with QTL for temperature depression and yield. Of the three loci on chromosome 1B, 3B and 5D that were associated with leaf cuticular wax content, cooler canopies and improved yield under heat stress in the greenhouse studies, two were detected in the field environments also. Presence of these loci or a combination of these loci may improve adaptation to high temperature stress.

CHAPTER V

CONCLUSIONS

Wheat cultivars with cooler canopies were tolerant to high temperature stress. Leaf cooling under high temperature conditions is a result of interaction of several physiological and morphological factors. Dissipation of excess heat and cooling of canopies may result from re-radiation, convective heat transfer or transpirational cooling. Based on the results of this study both transpiration and leaf cuticular wax content associated with canopy temperature depression. The significant correlation between stomatal conductance and leaf canopy temperature depression indicates transpirational cooling as primary heat adaptive mechanism. The presence of higher amount of leaf cuticular wax content increased reflectance and maintained a more effective control of water loss from plants and improved adaptation under high temperature stress. Leaf cuticular wax content increased in some cultivars during high temperature stress. Increase in flag leaf cuticular wax content in wheat in response to high temperature stress has not been reported.

Leaf cuticular waxes were associated with leaf temperature depression and HSI of yield components during short-term heat stress conditions, but failed to show any major associations during long-term heat stress. Although leaf cuticular wax content had low correlations with yield components but its positive association with leaf temperatures under both greenhouse and field conditions signifies its importance in cooling canopies.

QTL analysis detected QTL for leaf cuticular waxes, leaf temperature depression and yield components. Stable QTL for leaf cuticular waxes were detected on chromosome 1B and 5A. These QTL co-localized with QTL for temperature depression and yield. The QTL on chromosome 1B was consistent with a previously identified spike non-glaucousness loci. Of the three loci on chromosome 1B, 3B and 5D that were associated with leaf cuticular wax content, cooler canopies and improved yield under heat stress in the greenhouse studies, two were detected in the field environments also. Presence of these loci or a combination of these loci may improve adaptation to high temperature stress.

Although stable QTL for leaf cuticular wax content, canopy temperature depression and yield components were identified, mapping in a larger population may be essential to validate the QTL. Further the knowledge of various leaf cuticular wax genes in other crop species may be used to design specific primers for mapping purposes. Given the importance of leaf cuticular waxes in heat and drought tolerance and disease resistance further work is required to identify genes involved in the production and transport of leaf cuticular waxes.

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